STUDIES ON BRAIN ARYLSULPHATASES

(ARYLSULPHATE SULPHOHYDROLASE EC 3.1.6.1)

A THESIS
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GENERAL INTRODUCTION

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The sulphate esters present in the cells are hydrolysed by a group of enzymes, the sulphatases according to the general reaction.

$$R.O.\ SO_3^- + H_2O \longrightarrow R.\ OH + H^+ + SO_4^{2-}$$

The most thoroughly investigated group of sulphatases is the arylsulphatases. They catalyse the hydrolysis of arylsulphates or the sulphate monoesters of phenolic hydroxyl groups. They are widespread in their distribution and have been found in all animal species studied, in several micro-organisms, higher plants and in molluscs. Two groups of arylsulphatases type I and type II have been distinguished, by their substrate specificity, response to inhibitors and subcellular localization in Ox, Rat, and Human livers (1-3).

Type I Arylsulphatases:

The type I arylsulphatases are more active towards simple substrates such as p-nitrophenyl sulphate and p-acetylphenyl sulphate. They are much less active

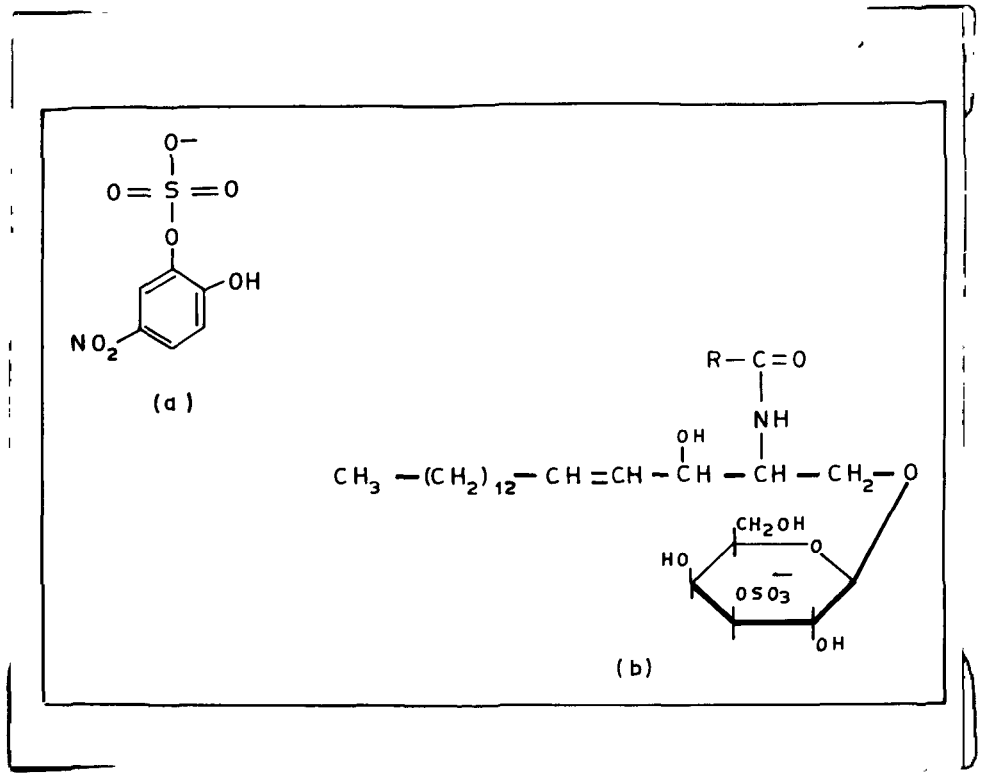

Fig.1. Structure of (a) p-Nitrocatechol sulphate and (b) Cerebroside 3-sulphate, in which R=the alkyl chain of a fatty acyl group.

towards p-nitrocatechol sulphate (Fig. 1). In general these enzymes are inhibited by sulphite, cyanide, phenylhydrazine and hydroxylamine but not by chloride, fluoride, phosphate and sulphate ions. The most detailed specificity studies have been made on the arylsulphatase of Alcaligenes metalcaligenes using a series of substituted phenyl sulphates (4). In this case it has been shown that the introduction of an electrophilic substituent into the phenyl sulphate nucleus increases both the rate of hydrolysis of the substrate and its affinity for the enzyme; the introduction of a nucleophilic substituent induces the opposite effects. The sulphatase of A. metalcaligenes furnishes an example of a relatively rare type of inhibition (anti-competitive) in which the inhibitor (cyanide or hydrazine in this case) combines with enzyme-substrate complex but not with the enzyme (5). This enzyme also hydrolyses tyrosine-O-sulphate, an unusual substrate as it is not split by mammalian arylsulphatase C, although theoretically it should be (6). Benkovic, Vergara and Hevey (7) purified an arylsulphatase from Aspergillus oryzae. The purified enzyme is inhibited by sulphite, fluoride and phosphate but not by sulphate, chloride and cyanide. On the basis of classification suggested by Dodgson and

Spencer (2) it is neither type I nor type II. At pH 4.8 p-nitrophenyl phosphate acts as a competitive inhibitor of the enzyme activity towards p-nitrophenyl sulphate. It is interesting to note, however, that at pH 7.5 p-nitrophenyl phosphate is not an effective inhibitor of either A. oryzae or A.aerogenes enzyme. Fowler and Rammeler (8) also reported that the phenol sulphatase of A.aerogenes does neither fall in type I nor type II category because it is inhibited by fluoride, cyanide and phosphate but not by sulphate ions. Apte and Siddiqi (9) also have purified an arylsulphatase from Aspergillus nidulans. The enzyme was resolved into two distinct fractions designated as fraction I and fraction II by DEAE-cellulose column chromatography. These fractions differ each other in their Km values for p-nitrophenyl sulphate and p-nitrocatechol sulphate, sensitivity to some inhibitors, electrophoretic mobility and heat stability. On the basis of their substrate specificity and sensitivity to sulphate, phosphate and cyanide both fractions can be classified as type I arylsulphatases.

The type I arylsulphatases of mammalian liver are generally inhibited by cyanide and sulphite ions but hardly affected by the phosphate and sulphate ions. The type I arylsulphatases of mammalian livers, the

arylsulphatases C, differ from the corresponding enzymes of micro-organisms in their extreme insolubility (10-12). They are localized in the microsomes (13-15) and very difficult to solubilise. The only soluble arylsulphatase C which has been obtained was prepared from rat liver microsomes by treatment with crude pancreatic enzymes in the presence of a non-ionic detergent (10). Similar preparations of the corresponding human (16) or Ox (12) enzymes could not be obtained. The hydrolysis of arylsulphates by the type I arylsulphatases involves fission of the O-S bond, as has been shown by the use of O^{18} enriched water (17), but there is little information on the nature of the active centres of these enzymes. Studies on the variation of Km with pH have shown that the active centres of the type I enzymes from Ox liver (12) and from A.metalcaligenes (18) contain grouping with pK values consistent with their being aminogroups, in agreement with the results obtained using specific inhibitors (18) which also suggest the occurrence of carbonyl group therein (18, 19).

Type II Arylsulphatases:

Type II arylsulphatases have been detected in plants, animal tissues and micro-organisms. The most studied

examples are the arylsulphatases A and B of mammalian tissues, some of which have been highly purified (20-25). Another group comprises the arylsulphatases of mollusc tissues (26-29). The mollusc arylsulphatases are seen to represent a transition between the type I enzymes and the type II of vertebrates, although they are definitely more closely related to the latter. In plants thoroughly investigated type II arylsulphatases are from the seeds of the mustard, Sinapis nigra (30). A type II arylsulphatase, very similar in its properties to a mammalian arylsulphatase B, has been isolated from Proteus vulgaris by Dodgson (31) and studied in some detail. This is the first example of a type II arylsulphatase in micro-organisms.

The type II arylsulphatases are characterised by their high activity towards p-nitrocatechol sulphate and much less activity towards simple substrates. These enzymes are strongly inhibited by sulphite, phosphate, sulphate, and fluoride ions but not by cyanide ions. The mammalian liver has been shown to contain a microsomal arylsulphatase C belonging to the type I and arylsulphatase A and B belonging to the type II (11,12,24). The enzymes arylsulphatase A and B are localised in lysosomes and can be readily solubilised.

They differ from each other in their reaction kinetics, pH optimum, affinity towards p-nitrocatechol sulphate and electrophoretic mobilities.

The kinetics of arylsulphatase A are extremely complex, because the reaction velocity shows abnormal relationship with the enzyme concentration and time of incubation (32). The anomalous reaction kinetic of arylsulphatase A is manifested as a time-dependent loss of hydrolytic rate during incubation with p-nitrocatechol sulphate, followed by a partial recovery of the initial rate. Baum and Dodgson (32) have proposed "during the interaction of enzyme and substrate a new site capable of binding with substrate as well as the reaction products (nitrocatechol and sulphate) and certain other inhibitory compounds is slowly exposed to the enzyme. When the substrate is bound to the new site of the modified enzyme the latter is virtually inactive. On the other hand when the reaction products or other inhibitory compounds are bound to the new site, the modified enzyme is active, although the active centre may still be inhibited if these compounds are present in excess". This hypothesis is recently confirmed by Nicholls and Roy (33) who actually isolated the so-called "inactive modified enzyme" from incubation

mixture. Apparently "inactive modified enzyme" is strongly activated by sulphate, but only slightly activated by nitrocatechol. Phosphate and pyrophosphate, the inhibitors of the native enzyme, also activate the modified enzyme. The low activity of the modified enzyme was found due to a powerful substrate inhibition which is decreased in the presence of sulphate. Further more it was postulated that sulphate displaces the equilibrium in favour of the native enzyme primarily through the formation of a "native enzyme-substrate complex". The involved kinetics of arylsulphatase A depend strongly upon the pH, the observed pH optima depend upon the time of incubation. In the case of human liver arylsulphatase A, Baum, Dodgson and Spencer (20) have reported two pH optima (pH 4.4 and 5.0) for short incubation periods which over long incubation periods or at higher enzyme concentrations, merge into a single pH optimum of 4.7. Balasubramanian and Bachhawat (34) and Harinath and Robins (35) reported only one pH optimum for human brain arylsulphatase A.

Distribution of Arylsulphatases:

The earlier investigation of this problem has been hampered by the lack of the methods of assaying these enzymes in unfractionated tissue preparations and

much of the published work is unreliable because of the lack of appreciation of the complexity of the problem. The most reliable information on the distribution of the arylsulphatases in mammalian tissues is that of Dodgson and his group for the rat (36) and the human (16). They have shown that, in general, liver is the organ richest in arylsulphatases and that considerable amounts also occur in the kidney, pancreas and adrenal. Arylsulphatases occur in human urine and serum also (37, 38, 39). The soluble arylsulphatase has also been found in epiphyseal, articular and rib cartilages and in metaphysis and bone marrow of rat (40).

Arylsulphatase activity in nervous tissue was first reported by Neuberg and Simon in rabbit brain (41). Arylsulphatase activity in homogenates of whole rat brain was reported by Dodgson, Spencer and Thomas (36). Balasubramanian and Bachhawat (42) studied the regional distribution of arylsulphatase activity in sheep brain and they found that the activity of arylsulphatases was high in those regions which were rich in white matter. Similar results on the regional distribution were obtained by Dzialoszynski and Wenclewski (43) in cow brain. The regional distribution of arylsulphatases in rat brain was studied by Sellinger and Hiatt (44).

In liver the intracellular distribution of arylsulphatases was studied by Roy (15) and Viala and Gianetto (45) according to these authors arylsulphatase A and B occur in lysosomes while arylsulphatase C is found exclusively in microsomes. These findings have been recently confirmed by cyto-chemical staining method (46). Clendenon and Allen (47) studied the subcellular distribution of arylsulphatases in rat brain and they found that the localization of arylsulphatases was the same as in the liver. Roy (48) has reported that arylsulphatase B of ox liver is localized in the framework of the lysosomes while arylsulphatase A is in the sap.

Arylsulphatases have been detected in all animal species (15, 49, 50). The quantitative data are available only for the liver. Here also the separate determination of arylsulphatase A and B has not been carried out, and hence proportion of these enzymes is only approximately known. Roy (15) has studied the proportions of arylsulphatase A and B by separating them on paper electrophoresis and according to him the enzymes corresponding to arylsulphatases A and B are detected in all the species except the guinea-pig, the hen, the frog and the stickleback. In frog and stickleback only arylsulphatase B is present. In the guinea-pig the situation

was quite different, although only one arylsulphatase was detected by paper electrophoresis, the shape of pH curve suggested that more than one such enzyme was present. In the hen also the pH curve suggested that both arylsulphatases A and B were present, although they could not be detected by paper electrophoresis. However, the arylsulphatases of the hen were apparently much less stable during electrophoresis than were those of the other animal species, as the total amount of activity recovered after electrophoresis was only a very small proportion of that originally present. This instability might well account for the apparent absence of arylsulphatase A from hen liver.

Further, it is of interest to compare the situation in the vertebrates with that in the few invertebrate species so far studied. Electrophoretic (29) and kinetic (26, 29) studies have shown that the arylsulphatase of the limpet, Patella vulgata, is comparable in many respects with a mammalian arylsulphatase A. Similarly in the snail, Helix pomatia, the arylsulphatase seems to be of the A type, as judged by its kinetic properties and its behaviour on electrophoresis (51). Dodgson, Lewis and Spencer (26) did not detect in the limpet any arylsulphatase C. Thus in the few invertebrates

so far studied the sole arylsulphatase present seems to be of the type of mammalian arylsulphatase A, in rather striking contrast with the situation in the lower vertebrates where the arylsulphatase is of the B type. It should be noted however, that the invertebrate sulphatases do not show the kinetic anomalies of mammalian arylsulphatase of the A type (32).

The livers of the marsupials Trichosurus vulpecula and Phascolomis mitchelli, the monotreme Echidna aculeata, the lizard Tiliqua rugosa and the frog Rana temporaria have also been studied with regard to their content of arylsulphatases (50). Type I arylsulphatases were found only in the opossum (T. vulpecula) and the lizard, in very small amounts in the latter. Type II arylsulphatases were found in all the species studied. The enzymes had the electrophoretic properties of arylsulphatase B from eutherian mammal but they were not typical of this group as their hydrolyses of p-nitrophenyl sulphate were not greatly activated by chloride ions and they seemed to combine many of the properties of the arylsulphatases A and B of the higher mammals. Thus type II arylsulphatases of lower mammals do not correspond to either arylsulphatase A or arylsulphatase B but rather combine some of the properties of both.

Regarding the mode of action of the type II arylsulphatases Spencer (17) has shown that it is the O-S bond which is splitable. It has been suggested that the active centre of rabbit liver arylsulphatase A probably contains an imidazole group (52). Quite recently, Jerfy and Roy (53) demonstrated by the use of several group specific reagents, that tyrosine and histidine residues are essential for the activity of arylsulphatase A from ox liver. They also reported that neither SH groups nor aminogroups are involved in the reaction catalysed by arylsulphatase A. The active centre of human arylsulphatase B contains grouping with pK values of 5.3 and 6.9 which might be imidazole residues (54). Bleszynski, Leznicki and Lewosz (55) suggested that β-aspartic and γ-glutamic carboxyles and imidazole may be involved in the active centres of arylsulphatase B. According to these authors although the structure of the active centre seems to be very similar for all soluble arylsulphatases of ox brain. The aminoacid composition of arylsulphatase A and B must differ to some extent as their UV-spectra are very different.

Arylsulphatases in pathological conditions:

Pronounced changes of the arylsulphatase activity

have been observed in pathological cases. Many diseases are associated with increased excretion of arylsulphatases in urine (56). A thirty-fold increase of arylsulphatase activity was observed in myeloid leukemia (57). Kwashiorkor patients have also shown increased urinary excretion of arylsulphatase A (58). A considerable increase of arylsulphatase activity in urine was observed in case of pulmonary and retinal tuberculosis (59) and in some cases of tumour (57).

Ugazio and his co-workers (60) studied the influence of carbon-tetrachloride, introduced intraperitonially, on the activity of various enzymes (among them arylsulphatase A and B) in fatty livers induced by that agent in the rat. The authors found increased specific activity of arylsulphatases in the liver homogenate at advance stage of poisoning.

It is suggested that elevated arylsulphatase activity in urine may be connected in some way with observed enlargement of the spleen (61). Various workers have found increased arylsulphatase activity in various tumours as compared with the activity in the parent tissue (62-64). Austin et al (65) found very low arylsulphatase A activity in the brain, kidney, liver and

urine of the patients with metachromatic leukodystrophy. A very marked elevation in arylsulphatase B activity was also observed in liver in gargoylism (65).

Arylsulphatase A:

Arylsulphatases A and B can be separated by fractional precipitation with acetone (22), paper electrophoresis (15), gel filtration with sephadex G-200 (66) and ion exchange chromatography (67).

Arylsulphatase A was purified from ox and human livers by Roy (23) and Baum, Dodgson and Spencer (20) but these preparations were not very pure. Moreover, the possibility of alteration in the structure of the enzyme could not be excluded, as these authors have used acetone during the enzyme purification.

Nichol and Roy (68) described a new purification procedure for arylsulphatase A of ox liver in which the conventional acetone fractionation was omitted, and instead the separation of arylsulphatases A and B was achieved by taking advantage of the fact that the arylsulphatase A will precipitate as an insoluble tetramer at acid pH. These workers (69, 70) have recently used the analytical ultracentrifuge in a detailed study of the

sub-unit structure of the enzyme. The molecular weight of the enzyme at neutral pH was estimated as 107000. The protein is very acidic with an iso-electric point at pH 3.4 and as the pH is lowered towards this value the monomer shows an increasing tendency to associate to produce a tetramer of molecular weight 411,000. Probably, hydrophobic bonds are involved in the aggregation since it is reversed by dioxan, as well as by raising the pH so that the electrostatic repulsion between similarly charged units increases. Conversely treatment of the monomeric units with sodium dodecyl sulphate causes them to break down to components of 24,000 molecular weight which, on the basis of chemical data, may be of more than one kind.

Recently Roy (71) has purified arylsulphatase A from Kangaroo liver. This arylsulphatase A is different from ox liver arylsulphatase A in the sense that it has molecular weight of 100,000, does not polymerise at low pH, has an isoelectric point 5.1 - 5.4 and shows less pronounced anomalous kinetics. Balasubramanian and Bachhawat (34) have purified an arylsulphatase from human brain which resembles arylsulphatase A of human liver. The extraction and purification of arylsulphatase A and its properties from ox and human brain have been recently

reported (35, 72, 73). The method for the preparation of an immunoelectrophoretically pure arylsulphatase A from human placenta has also been recently described (74).

Arylsulphatase A and metachromatic leukodystrophy:

According to Roy (3) arylsulphatases are unlikely to be active in living tissues because they would be inhibited by the normal concentrations of phosphate and chloride, even if they had natural substrates but Flynn et al (75) have shown the hydrolysis of (^{35}S) p-nitrocatechol sulphate in perfused rat liver, or anaesthetised rats in which kidney function had been eliminated by ligature of the renal pedicles. It may be that the special micro-environments that exist for the functioning of lysosomal enzymes e.g. the endocytotic vacuoles, are adapted to the requirements of arylsulphatases. The identification of arylsulphatase A as cerebroside sulphatase was made in two ways. Patients with metachromatic leukodystrophy exhibit accumulation of cerebroside 3-sulphate (Fig. 1). in various organs and spillage into urine, from this it seemed likely that the cerebroside 3-sulphate degrading enzyme might be lacking in such individuals. In a series of pioneering studies, Austin (76) showed that these individuals had typical arylsulphatase B activities but little or no arylsulphatase A

activity. Mehl and Jatzkewitz (77) found a lack of activity towards cerebroside 3-sulphate in human leukodystrophic kidney. Moser et al (78) studied the turnover of cerebroside 3-sulphate in human subjects and found that the leukodystrophic subjects exhibited very slow break down of cerebroside 3-sulphate.

A more direct comparison has been made with highly purified arylsulphatase of kidney (79). Electrophoresis yielded two peaks one for the A enzyme and one for the B enzyme. The distribution of the cerebroside sulphatase activity after electrophoresis was found to parallel with the distribution of arylsulphatase A to some extent, although not exactly. This discrepancy was explained by the incomplete separation of arylsulphatase A from so called complementary "heat stable fraction", which moves little ahead of peak A, probably overlapping it. They have further shown that this heat stable material greatly stimulates the cerebroside sulphatase activity although it has no effect on arylsulphatase B activity. The role of this material has not been worked out.

The cerebroside sulphatase activity (80) is inhibited by sulphate, sulphite, phosphate, pyrophosphate and fluoride but not by cyanide, in these respects

resembling arylsulphatase A (2). Furthermore cerebroside 3-sulphatase activity was also inhibited by cerebroside 6-sulphate, galactose 3 or 6-sulphates (79). These findings support the hypothesis of Austin et al (65, 81) who demonstrated that the tissues of patients with metachromatic leukodystrophy contained exceptionally low arylsulphatase A, from this they postulated that the lysosomal enzymes may be essential for the normal turnover of these sulphated glycolipids which accumulate in the congenital disease.

Very recently Jatzkewitz and Mehl (82) have demonstrated that both arylsulphatase A and cerebroside 3-sulphatase activities were reduced to the limit of detection in the several tissues of metachromatic leukodystrophy patients. Taninguchi and Nanba (83) studied arylsulphatase A activity in the leukocytes from patients had from their close relatives in three families with metachromatic leukodystrophy. The activity of arylsulphatase A in leukocytes of metachromatic leukodystrophy was approximately one-tenth of the control value. The enzyme activity in leukocytes from the parents of affected patients and from some of their relatives was approximately half that found in control subjects. There appeared to be no overlap between the enzyme activities in leukocytes

from the parents of patients and from normal individuals. The deficiency of arylsulphatase A in metachromatic leukodystrophy was also confirmed by the use of skin fibroblast obtained from the metachromatic leukodystrophy patients (84). The cultured fibroblasts derived from patients with late infantile metachromatic leukodystrophy incorporated arylsulphatase A from the growth medium. Upon exposure to cerebroside sulphate, the fibroblasts exhibited patterns of uptake and hydrolysis indistinguishable from cells derived from control subjects. Furthermore, inclusion granules formed in the metachromatic leukodystrophy fibroblasts upon exposure to cerebroside 3-sulphate were cleared by subsequent supplementation of the growth medium with arylsulphatase A (85).

Recently Porter et al (86) studied a correlation of intracellular cerebroside sulphatase activity in fibroblasts with latency in the metachromatic leukodystrophy and reported that despite the absence of cerebroside sulphatase activity in cell-free preparations, fibroblasts in culture derived from patients with metachromatic leukodystrophy, were capable of hydrolysing exogenous cerebroside sulphate. Moreover, the degree of whole cell arylsulphatase activity was directly correlated to the age of onset of clinical symptoms in the patients

from whom the fibroblasts were derived. In fact fibroblasts from patients with the earliest manifesting form, late infantile metachromatic leukodystrophy, did not hydrolyse and cerebroside sulphate, while fibroblasts from patients with later manifesting forms hydrolysed appreciable amounts of the sulpholipid.

Neuwelt et al (87) have reported that metachromatic leukodystrophy tissue extracts contain immuno-reactive proteins which precipitate anti-arylsulphatase A serum. These findings and the data of Porter et al (86) with fibroblasts provide evidence that arylsulphatase A proteins are produced in some types of metachromatic leukodystrophy. These mutant proteins appear to be similar in that they all exhibit extreme lability under normal extraction procedures but they may differ among themselves in affinity for cerebroside sulphate or in some other parameter which determines latency of clinical onset. The studies by Porter et al (86) and the wide range of reported latencies in clinical manifestations suggest that the metachromatic leukodystrophies result from a broad spectrum of cerebroside sulphatase deficiency ranging from virtual absence to an appreciable percentage of normal levels in those cases of late onset of symptoms.

The studies by Koenig and his co-workers (88) have indicated that essentially all the enzyme proteins of rat kidney and liver lysosomes are glycoproteins and that at least some of these glycoproteins contain sialic acid (88). Recently Goldstone, Konecny and Koenig (89) have claimed that approximately 40% of arylsulphatase A can be converted to arylsulphatase B by neuraminidase treatment and the incubation without neuraminidase produced a smaller conversion of the arylsulphatase A into arylsulphatase B. The arylsulphatase B produced by neuraminidase treatment showed increased binding affinity for p-nitrocatechol sulphate and reacted as arylsulphatase B on biochemical assay (90). But it is really surprising because arylsulphatase A has more affinity for p-nitrocatechol sulphate and arylsulphatase B has less affinity for this synthetic substrate. On the basis of the acidic and basic nature of lysosomal hydrolases the above authors have proposed the deficiency of the specific sialyl transferase which produces arylsulphatase A, deficient in metachromatic leukodystrophy, from arylsulphatase B which is present in normal or elevated amounts in this disease.

Arylsulphatase B:

Earlier described preparations (24) of arylsulphatase B were grossly impure. Recently Allen and Roy (91)

have described a new method for the preparation of arylsulphatase B from ox liver. The enzyme was resolved into two fractions, arylsulphatase Bα and Bβ by CM-sephadex chromatography. Each fraction was purified about 2000-fold and obtained in a yield of about 5%. Both fractions have molecular weights of about 25,000 and they are kinetically indistinguishable. The purified enzyme was examined by the technique of isoelectric focussing and it was shown that during this procedure the arylsulphatase B aggregates to mixtures of polymers having molecular weights of upto at least 300,000. The aggregation is probably due to electrostatic interaction because it is reversed by increasing the ionic strength. Arylsulphatase B of human liver was also purified (54). This enzyme had little activity towards potassium phenyl sulphate and its monosubstituted derivatives, but it showed appreciable activity towards disubstituted derivatives. Arylsulphatase B from ox brain (55,72) and human brain was purified by Bleszynski and Harinath and Robins (35). The ox brain arylsulphatase B was resolved into three subfractions by DEAE-cellulose chromatography and these subfractions were designated as B-1, B-2 and B-3. According to Bleszynski (73) these subfractions were similar to Wortman's fractions (92) 'a', 'b' and 'c'

which he obtained from ox cornea.

Arylsulphatase B too is not without its anomalies. This enzyme hydrolyses p-nitrocatechol sulphate, but has often been considered quite without activity against p-nitrophenyl sulphate. Webb and Morrow (25) however, found that a partially purified arylsulphatase B from ox liver hydrolysed p-nitrophenyl sulphate when activated by chloride. On the other hand, the hydrolysis of p-nitrocatechol sulphate by the same enzyme was inhibited by chloride, at high concentrations. The activity against p-nitrophenyl sulphate was inhibited by phosphate, sulphate, and fluoride but not through competition with chloride; the two may have competed with the substrate. Ox liver arylsulphatase B has low affinity for p-nitrocatechol sulphate and is inhibited by citrate (24). Allen and Roy (91) have recently confirmed the requirement of chloride for the hydrolysis of p-nitrophenyl sulphate that was first described by Webb and Morrow (25).

Arylsulphatase B and Mucopolysaccharidoses:

The role of arylsulphatase B is quite obscure. However, a marked increase in arylsulphatase B activity was observed in Hurler's syndrome (93). Further it

was found that although there was increase in heparan sulphate and dermatan sulphate fraction in Hurler's disease compared to normal, the total mucopolysaccharide content in Hurler's and normal brain was the same. Recently Abraham et al (94) reported that there was a marked increase of arylsulphatase B in the biopsy samples of patients with Sanfilippo syndrome compared to arylsulphatase A and the ratio of arylsulphatase B to A was very high compared to that of the normal brain.

Held and Buddecke (95) using (^{35}S) chondroitin 4-sulphate of bovine nasal septum, have purified chondroitin 4-sulphatase free from arylsulphatase and hyaluronidase from bovine arterial tissue and showed that this is a specific enzyme acting at polymer level. The enzyme has pH optimum at 4.4 in acetate buffer. The release of inorganic sulphate from unlabelled chondroitin 4-sulphate could be demonstrated turbidimetrically by converting it to barium sulphate, but this method did not show any action of the enzyme on chondroitin 6-sulphate, dermatan sulphate or keratan sulphate. The above authors also claimed that the addition of hyaluronidase did not stimulate the release of sulphate by this enzyme. Very recently Tudball and Davidson (96) have shown the existence of an enzyme related to that of bovine aorta

and yet distinct in lysosomes of rat liver. Although partially purified, enzyme was contaminated with β-glucuronidase, β-acetylglucosaminidase and arylsulphatase; its substrate specificity was curious in that it was most active with high molecular weight oligosaccharides derived from (^{35}S) chondroitin 4-sulphate of chick embryo by the action of hyaluronidase. The enzyme had less action on low molecular weight oligosaccharides and none on the parent polysaccharide; the removal of the non-reducing glucuronosyl residue of the oligosaccharide substrate was apparently essential for enzymic activity. Lloyd (97) and his associates have shown that mammalian sulfamidase librates inorganic sulphate from heparan sulphate by the cleavage of sulphamido groupings. Recently, a sulphamidase has been purified from mammalian sources by Dietrich (98).

Assay of Arylsulphatases A and B:

There have been difficulties in assaying arylsulphatases A and B when they are present together, as in human urine. A solution to this problem has been worked out by Baum, Dodgson and Spencer (99) by utilising a differential inhibition with chloride ions and other small differences in properties. The determination

of arylsulphatase A in the presence of B is based upon the following principles:

1. 'Normal Kinetics' are exhibited by arylsulphatase A at low substrate concentration in the presence of 2.5×10^{-4} M-sodium pyrophosphate.

2. Considerable inhibition of arylsulphatase B occurs under these conditions.

3. Arylsulphatase B is specifically inhibited by chloride ions when p-nitrocatechol sulphate is used as substrate, whereas arylsulphatase A is not.

The determination of arylsulphatase B in the presence of A is based on the fact that arylsulphatase A exhibits only a little residual activity after the first 20 minutes of the reaction when incubated at high substrate concentration in the presence of barium ions and in the absence of interfering ions. Moreover, this residual activity is linear and proportional to the intercept obtained by extrapolating this line back to zero time. Barium ions do not affect the activity of arylsulphatase B. According to Dodgson and his co-workers (67) this assay procedure may not be applied to rat liver arylsulphatases A and B because the differences in the kinetic properties of these enzymes are not sufficiently great to enable the independent assay of one enzyme in the presence of other.

AIMS AND OBJECTS OF THE PRESENT STUDY.

It is clear from the general introduction that there is a good relationship between arylsulphatases and cerebroside 3-sulphate and mucopolysaccharides. The importance of mucopolysaccharides in the synthesis of myelin in brain has been indicated by various workers (100, 101, 102). The sulphur containing lipids, cerebroside 3-sulphate are the components of the myelin sheaths of the nervous system. As a building-blocks of the myelin they are of great importance and any abnormal changes in their concentration result in extensive damage to the myelin as seen in the disease metachromatic leukodystrophy which is characterised by the deficiency of arylsulphatase A not only in brain and kidney but also in urine (65). Further, Austin et al (103) and Saxena et al (104) have reported that arylsulphatase A levels tend to parallel the level of incorporation of (^{35}S) sulphate into cerebroside 3-sulphate of the rat brain and the levels of arylsulphatase A and cerebroside 3-sulphate reach their peak in young rat brain during myelination period. A study of cerebroside sulphotransferase activity in the developing rat brain in our laboratory (104) has revealed that there was practically no cerebroside 3-sulphate synthesising

activity upto 9 days after birth and there was a peak of activity around 18 to 22 days after birth once again emphasizing the fact that cerebroside 3-sulphate synthesis is maximum at the myelination period. Moreover, a very striking relationship between arylsulphatase activity and cerebroside 3-sulphate content of some regions of the sheep brain was also observed in our laboratory (42). Recently Mehl and Jatzkewitz (79) have shown that cerebroside 3-sulphate is a naturally occurring substrate for arylsulphatase A from pig kidney. However, at present there is no information available as to whether or not the brain arylsulphatase A can degrade cerebroside 3-sulphate.

In the light of these observations a systematic comparative study of arylsulphatases A and B activity was made in the brains of various animal species and also in developing rat brain. The enzyme arylsulphatase A was purified from chicken brain to study its physicochemical and kinetic properties. Some kinetic properties of this purified enzyme were also studied using cerebroside 3-sulphate as substrate.

CHAPTER II.

THE REGIONAL DISTRIBUTION, AGE DEPENDENT VARIATION AND SPECIES DIFFERENCES OF BRAIN ARYLSULPHATASES

CHAPTER II.

THE REGIONAL DISTRIBUTION, AGE DEPENDENT VARIATION AND SPECIES DIFFERENCES OF BRAIN ARYLSULPHATASES

1. Introduction:

There are several lines of evidence indicating a relationship between arylsulphatases and mucopolysaccharides and cerebroside 3-sulphates. The occurrence and importance of mucopolysaccharides and cerebroside 3-sulphates in brain have already been established (100,101, 105,106). A significant decrease in arylsulphatase A activity and accumulation of cerebroside 3-sulphates was observed in various tissues of metachromatic leukodystrophy patients (81,105). Mehl and Jatzkewitz (79) have recently demonstrated that cerebroside 3-sulphate is naturally occurring substrate for arylsulphatase A. Balasubramanian and Bachhawat (42) have reported a relationship between high arylsulphatase activity and cerebroside 3-sulphate content of some regions of brain. Collective evidence thus indicates that arylsulphatase A may have a role in regulating the cerebroside 3-sulphate content in brain. A marked increase in arylsulphatase B activity compared to A was observed in Hurler's disease (65,93). Furthermore, Abraham et al (93) found that

although there was an increase in heparan sulphate and dermatan sulphate fraction in Hurler's disease compared to normal, the total mucopolysaccharide content in Hurler's and normal brain was the same.

This chapter describes (a) the determination of arylsulphatase A and B in brains of various animal species, (b) the partial separation of arylsulphatases A and B of brain by fractionation with zinc acetate and comparison of properties of arylsulphatase A from avian and mammalian species (c) the determination of arylsulphatases A and B in different regions of monkey brain and (d) the variation of arylsulphatase A and B activities in developing rat brain.

2. Materials:

Dipotassium salt of p-nitrocatechol sulphate was purchased from Sigma Chemical Co., U.S.A. Acrylamide and N-N-methylene bis acrylamide were obtained from Eastman Organic Chemicals, Distillation Products Industries, U.S.A., Triton X-100 was purchased from Rohm and Hass, Philadelphia, Pa. Coomassie Brilliant Blue was obtained from Sigma Chemical Co;, U.S.A. and all other chemicals used were of analytical grade.

3. Methods:

(i) The brains of various animal species studied were taken out after decapitation, immediately chilled in ice and were homogenised with 2 ml/gm of 0.05M- tris-HCl, pH 7.4 in a Potter-Elvehjem homogeniser for 2 min. at $0^{o}C$ and homogenates were centrifuged at 800 X g for 30 min. the supernatant fluid suitably diluted, was used for the assay of enzyme activity. Since the original supernatant was diluted 40 times, the possibility of inhibition by phosphate ions present in the supernatant fluid was negligible.

3.(ii) Arylsulphatase assay: Arylsulphatases A and B were assayed by the method of Baum, Dodgson and Spencer (99) with slight modification. Assay mixture for arylsulphatase A:

0.1 ml of Reagent 'A' -
(0.01M-p-nitrocatechol sulphate in 0.5M-sodium acetate - acetic acid buffer containing 5 X 10^{-4}M-sodium pyrophosphate and 10% (w/v) NaCl pH 5.0).

0.1 ml of 1% Triton X-100 and
Enzyme 0.1 ml in a total volume of 0.3 ml.

Assay mixture for arylsulphatase B:

0.1 ml of Reagent 'B' -
(0.05M-p-nitrocatechol sulphate in

0.5M-sodium acetate-acetic acid
buffer containing 10^{-2}M-barium
acetate pH 6.0).

0.1 ml of 1% Triton X-100 and
Enzyme 0.1 ml in a total volume of 0.3 ml.

For arylsulphatase A after incubation at 37°C, for 1 h the reaction was stopped by the addition of 2.7 ml of 0.11M-NaoH and the red colour developed was measured in a Klett-Summerson Colorimeter using 50 filter. For arylsulphatase B the tubes were incubated at 37°C for 30 min. and 90 min. and the reaction was stopped as indicated above. The amount of nitricatechol formed was calculated for 1 h as described by Baum, Dodgson and Spencer (99). The specific activity was expressed as umoles nitrocatechol formed/mg protein/h.

3.(iii) Partial separation of arylsulphatases A and B: Frozen brain was homogenised with 2 volumes of 0.03M-tris HCl pH 7.4 for 2 min. in a Waring blender. The homogenate was cooled at -5°C in a bath of ethylene glycol and then subjected to ethanol fractionation with constant mechanical stirring. Ethanol 20% (v/v) was added dropwise and after 10 min. the mixture was centrifuged at 12,000 X g for 1 h at -5°C. A small portion (app. 5 ml) of supernatant was dialysed against 100 vol. of 0.001M-sodium acetate buffer pH 5 containing 0.0001M-$MgCl_2$ for 8 h.

The rest of the supernatant was subjected to zinc acetate fractionation. Zinc acetate solution (0.2M) was added to a final concentration of 0.005M at -5°C with constant mechanical stirring. After 15 min. the mixture was centrifuged at 12,000 X g for 1 h at -5°C. The reddish precipitate was dissolved in 0.1M-sodium citrate buffer pH 7 and dialysed for 4 h against 500 volumes of 0.001M-sodium acetate buffer pH 5 containing 0.0001M-$MgCl_2$. To the supernatant, zinc acetate was added to a final concentration of 0.02M. After 15 min. it was centrifuged at 12,000 X g for 1 h at -5°C. The supernatant was discarded and the white precipitate was dissolved in 0.1M-sodium citrate buffer pH 7 and dialysed as above.

3.(iv). Gel electrophoresis: Polyacrylamide gel-electrophoresis was carried out by the method of Ornstein and Davis (107) using Buchler instrument in 0.03M-sodium barbitone buffer pH 8.0 for 10 h at 8mA/tube. The proteins were stained by Coomassie brilliant blue (108). For elution of protein, the whole gel was cut into 1 cm segments and from each segment the protein was eluted by homogenising the gel in a Potter-Elvehjem homogeniser with 0.03M-tris-HCl, pH 7.4. The homogenate was centrifuged at 0°C. The supernatant after dialysis against 0.001M-sodium acetate buffer pH 5.0 containing

0.0001M-$MgCl_2$ was analysed for arylsulphatase A and B activities.

3.(v). Protein determinations: Protein was determined by the method of Lowry et al (109) with crystalline bovine serum albumin as a standard.

4. Results:

4.(i) The proportion of arylsulphatases A and B in brains of various animal species: Table I shows that in a lower vertebrate like frog the ratio of arylsulphatase A:B is 1:1. In birds, like pigeon and chicken arylsulphatase A accounts for greater part of the total arylsulphatase activity. In fact in chicken the activity of arylsulphatase A is exceptionally high compared to B. Further in the case of birds arylsulphatase A is present in large amounts not only in brain but also in other organs like liver and kidney (Table II). In mammals like rabbit and sheep arylsulphatase A is high while in mammals like rat, monkey and man arylsulphatase B predominates. Zinc acetate fractionation results in partial separation of arylsulphatases A and B in all species studied (Table III). It should be noted here that arylsulphatase B (0.005M-zinc acetate precipitate) was contaminated with 6 to 18% of arylsulphatase A, and arylsulphatase A

CHAPTER II

TABLE I

RATIO OF SPECIFIC ACTIVITIES OF ARYLSULPHATASE A AND B IN BRAINS OF VARIOUS ANIMAL SPECIES

Species	Specific activity A	Specific activity B	Ratio of specific activity A:B
Rat	0.28	0.59	1:2
Man (adult)	0.1	0.16	1:1.6
Child (2 years)	0.06	0.07	1:1
Hurler's syndrome patient (7 years)	0.03	0.17	1:5.6
Sanfilippo syndrome patient (8 years)	0.02	0.09	1:4.5
Monkey	0.07	0.12	1:1.7
Sheep	0.16	0.06	2.6:1
Rabbit	0.08	0.06	1.3:1
Pigeon	0.07	0.03	2.3:1
Chicken	0.1	0.003	33.3:1
Frog	0.02	0.02	1:1

The activities of arylsulphatase A and B were measured as indicated in Methods.

CHAPTER II

TABLE II

RATIO OF SPECIFIC ACTIVITIES OF ARYLSULPHATASE A AND B IN DIFFERENT TISSUES OF CHICKEN AND PIGEON

Tissues	Specific activity A	Specific activity B	Ratio of specific activity A:B
Chicken liver	0.07	0.009	7.7:1
Chicken kidney	0.13	0.020	6.5:1
Pigeon liver	0.34	0.090	3.7:1
Pigeon kidney	0.06	0.040	1.5:1

The activities of arylsulphatase A and B were measured as indicated in the Methods.

TABLE III.

PARTIAL SEPARATION OF ARYLSULPHATASE A AND B FROM SHEEP, CHICKEN, MONKEY, HUMAN AND RAT BRAIN.

Enzyme fraction	Total volume (ml)	Total protein (mg)	ARYLSULPHATASE A			ARYLSULPHATASE B		
			Total enzyme units*	Specific activity	Yield (per cent)	Total enzyme units*	Specific activity	Yield (per cent)
Sheep:								
Homogenate	266	9842	400	0.04	100	200	0.02	100
Extract (20% Ethanol supernatant)	200	3000	240	0.08	60	192	0.06	96
Zinc acetate fraction I (0.005 M)	53	1961	27	0.01	7	152	0.07	76
Zinc acetate fraction II (0.02 M)	29	696	177	0.25	44	20	0.02	10
Chicken:								
Homogenate	112	2128	100	0.05	100	3.7	0.002	100
Extract (20% Ethanol supernatant)	64	1312	73	0.06	73	3.7	0.003	98
Zinc acetate fraction I (0.005 M)	17	586	71	0.12	71	0.0	0.000	0
Zinc acetate fraction II (0.02 M)	12	213	16	0.07	16	2.6	0.012	69
Monkey:								
Homogenate	200	5000	260	0.05	100	240	0.05	100
Extract (20% Ethanol supernatant)	100	1460	186	0.13	71	100	0.07	42
Zinc acetate fraction I (0.005 M)	40	1120	44	0.04	17	88	0.08	37
Zinc acetate fraction II (0.02 M)	17	367	129	0.35	50	4	0.01	18
Human:								
Homogenate	65	975	51.0	0.052	100	58.7	0.060	100
Extract (20% Ethanol supernatant)	50	430	27.0	0.056	53	39.0	0.080	66
Zinc acetate fraction I (0.005 M)	19	380	5.7	0.010	11	28.5	0.075	48
Zinc acetate fraction II (0.02 M)	9	90	13.5	0.150	26	-	-	-
Rat:								
Homogenate	46	1840	552	0.30	100	516	0.44	100
Extract (20% Ethanol supernatant)	35	995	420	0.50	76	330	0.53	34
Zinc acetate fraction I (0.005 M)	21	357	99	0.27	18	194	0.54	23
Zinc acetate fraction II (0.02 M)	17	95	199	2.30	36	100	1.00	12

All fractions were assayed after dialysis.
*One unit of enzyme is defined as the amount of enzyme required to liberate 1 μmol of nitrocatechol from nitrocatechol sulphate in 1 h under usual assay conditions.

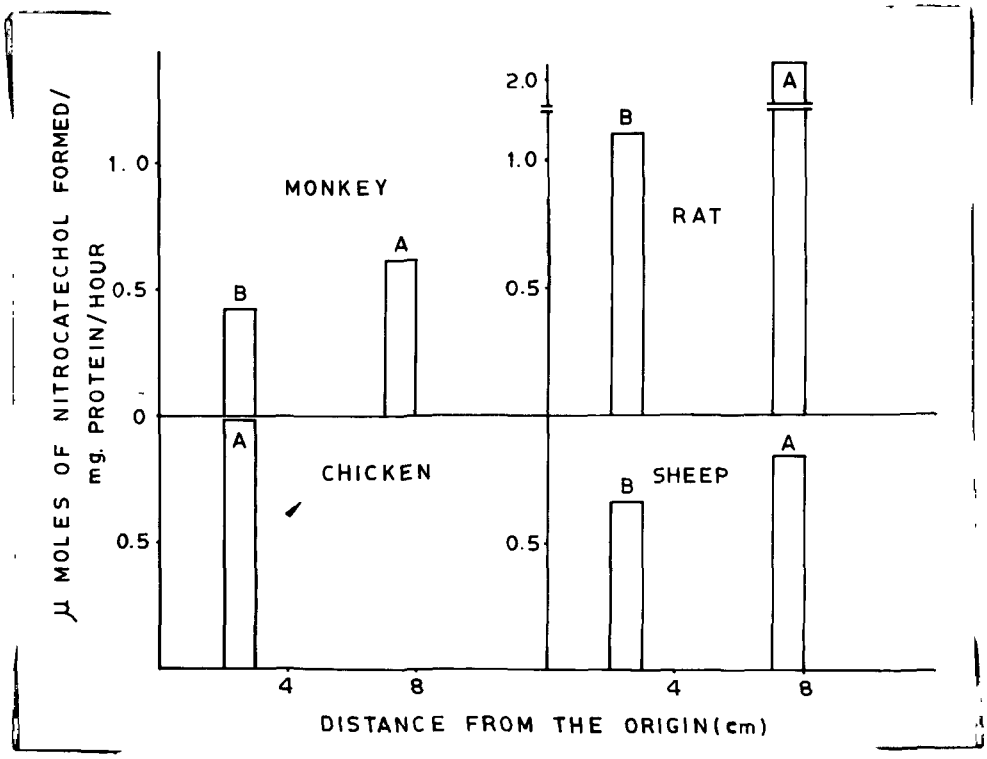

FIG.1. The electrophoretic mobility of monkey, rat, chicken and sheep brain arylsulphatases A and B. 7.5% Polyacrylamide gel in a 17 X 1 cm tube was used. The conditions for electrophoresis and the determination of arylsulphatase A and B activities were the same as indicated in the Methods.

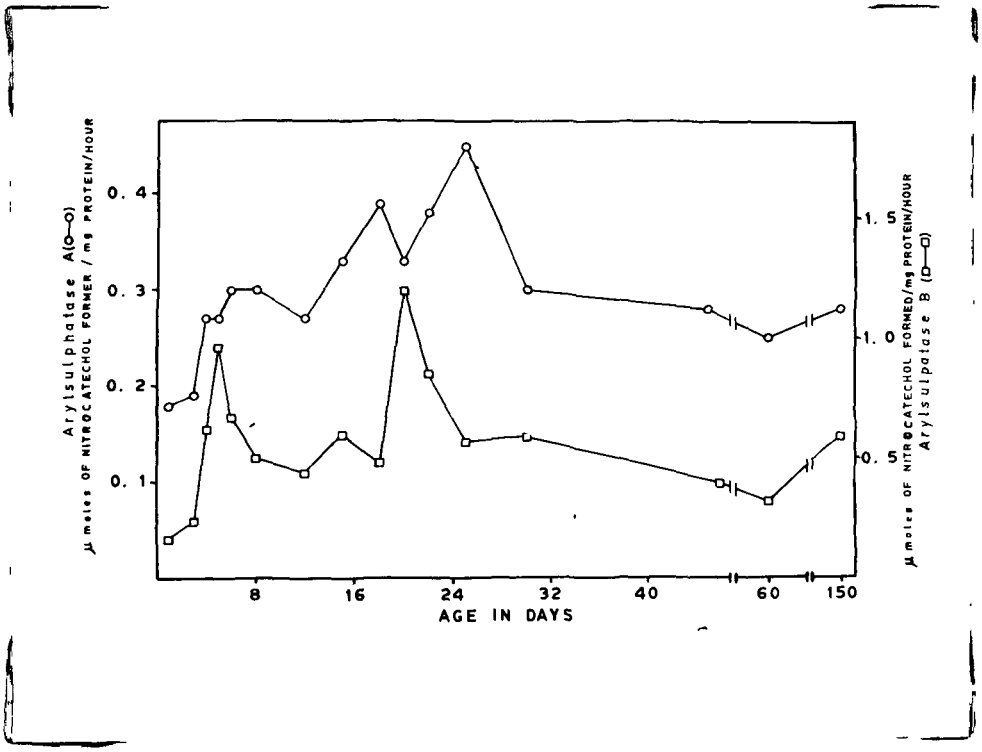

FIG.2. Variation of arylsulphatase A and B activities in developing rat brain. The activities were estimated as indicated in the Methods.

(0.02M-zinc acetate precipitate) was contaminated with 1.8 to 12% of arylsulphatase B. In the case of human brain, arylsulphatase A obtained by this procedure was found to be free from arylsulphatase B.

It is interesting to note that arylsulphatase A of chicken brain is quite different from arylsulphatase A of other species since under zinc acetate fractionation most of chicken arylsulphatase A precipitates out at a concentration of 0.005M while in other cases arylsulphatase A precipitates out only at a concentration of 0.02M. Furthermore, the electrophoretic mobility of chicken brain arylsulphatase A is similar to that of arylsulphatase B of other species but quite different from arylsulphatase A of other species (Fig.1). This finding is comparable with the finding of Roy (17) who has indicated the presence of both arylsulphatases A and B in hen liver although arylsulphatase A could not be detected on paper electrophoresis. It is clear from the above observation that chicken arylsulphatase A behaves like arylsulphatase B under zinc acetate fractionation as well as in electrophoresis. This may explain why Roy (17) was unable to detect the presence of arylsulphatase A electrophoretically in a crude extract of hen liver. Zinc acetate fraction I, and II

from rat, monkey, sheep and chicken brain were subjected to gel electrophoresis (Fig.1). The arylsulphatases A and B eluted from the gel were found to be free from each other. The increase in specific activity and recovery of arylsulphatases A and B are shown in Table IV.

4.(ii) Arylsulphatase A and B activities in developing rat brain: It appears from Fig.2 that arylsulphatase A and B activities are present in rat brain on the first day after birth and there is an increase in enzyme activity with age. It is interesting to note that there are two prominent peaks of arylsulphatase B corresponding to 5 and 20 days. The activity of arylsulphatase A is also high during myelination period.

4.(iii) Regional distribution of arylsulphatase A and B activities in monkey brain: The data on regional distribution (Table V) suggest that arylsulphatases A and B are present in all the regions of monkey brain. It is apparent that the activity of arylsulphatase A is high in white matter not only in cerebrum but also in regions like medulla and corpus callosum. The activities of arylsulphatase B are high in grey matter compared to white matter in most of the regions.

CHAPTER II

TABLE IV

RECOVERY AND SPECIFIC ACTIVITY OF ARYLSULPHATASE A AND B BEFORE AND AFTER ELECTROPHORESIS

Arylsulphatases from various species	Specific activity		Recovery (per cent)
	Before electrophoresis	After electrophoresis	
Sheep arylsulphatase A	0.254	0.84	32
Sheep arylsulphatase B	0.070	0.66	48
Chicken arylsulphatase A	0.120	1.00	46
Monkey arylsulphatase A	0.350	0.61	37
Monkey arylsulphatase B	0.078	0.47	38
Rat arylsulphatase A	2.300	2.60	38
Rat arylsulphatase B	0.540	1.10	42

Zinc acetate I and II fraction from various animal species were subjected to the gel electrophoresis. The conditions for electrophoresis are as described in the Methods. After elution from the gel, the activities of arylsulphatase A and B were estimated as indicated in the Methods.

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TABLE V

RATIO OF SPECIFIC ACTIVITIES OF ARYLSULPHATASE A AND B IN DIFFERENT REGIONS OF MONKEY BRAIN

Regions	Specific activity		Ratio of specific activities A:B
	Arylsulphatase A	Arylsulphatase B	
Cerebrum:			
Frontal grey	0.04	0.07	1:1.75
Frontal white	0.10	0.09	1.1:1
Parietal grey	0.05	0.11	1:2.2
Parietal white	0.05	0.12	1:2.4
Occipital grey	0.08	0.15	1:1.8
Occipital white	0.10	0.08	1.2:1
Cerebellum	0.04	0.06	1:1.5
Quadrigeminal bodies	0.07	0.16	1:2.2
Pons	0.10	0.14	1:1.4
Medulla	0.12	0.08	1.5:1
Corpus Callosum	0.09	0.06	1.5:1

Activities of arylsulphatase A and B were measured as indicated in the Methods.

4.(iv) Properties of arylsulphatases A and B in brains of different species:

pH optimum: Fig. 3 and 4 show the pH activity curves of arylsulphatases A and B of various animal species. The pH optimum for arylsulphatase A of rat, monkey, sheep and chicken was found to be 5.0, 4.5, 5.5 and 4.5 respectively in sodium acetate buffer. The reported optimum pH for human brain arylsulphatase A is 4.5 (34). The pH optimum for arylsulphatase B of rat, monkey and sheep in acetate buffer was 5.5, 5.5 and 4.5 respectively. When tested with zinc acetate fractions I and II, Triton X-100 had no effect on arylsulphatase A and B activities of various animal species.

The effect of the length of the incubation period: The time-activity curves (Figs. 5,6,7 & 8) for arylsulphatase A from various animal species show anamolous time-activity relationship (32,34) at various protein concentrations. It should be noted in the case of chicken arylsulphatase A the last two stages (stage II and stage III) are less pronounced compared to other species. Moreover, at high enzyme concentration in all the species stage II is not maintained and the curves tend towards a straight line. Arylsulphatase A of various animal species follows normal time-activity

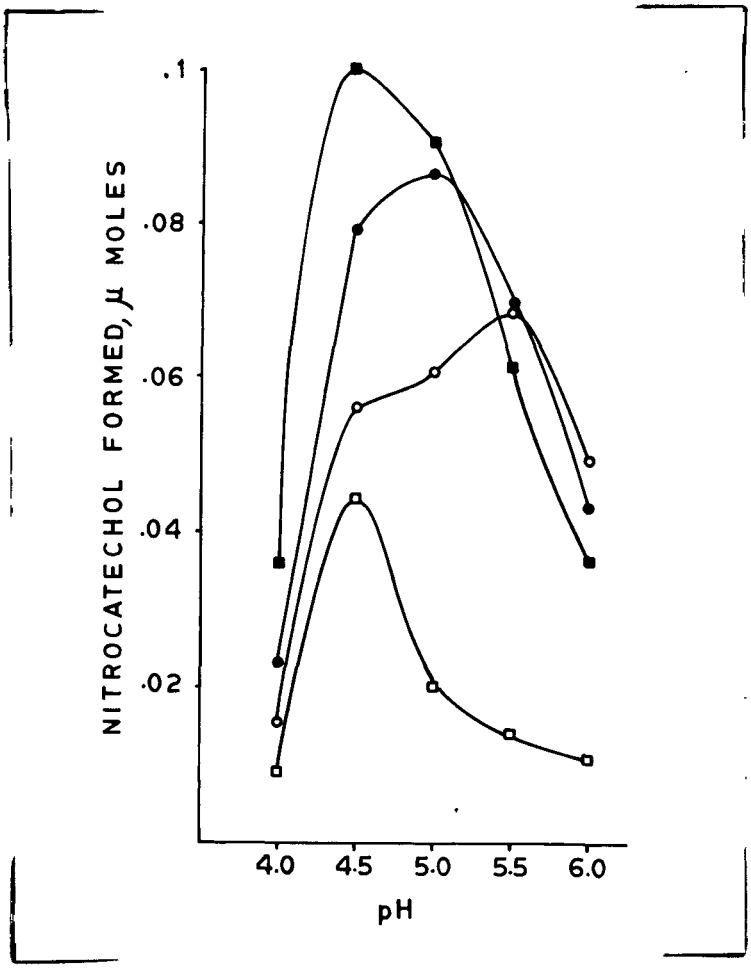

FIG.3. pH activity curves of rat, monkey, sheep and chicken arylsulphatase A. The incubation mixture consisted of 50 μmol of sodium acetate buffer of various pH values, 1 μmol of p-nitrocatechol sulphate, and enzyme (zinc acetate fraction II except in the case of chicken where zinc acetate fraction I was used) in a total volume of 0.3 ml. The reaction mixture was incubated at 37°C for 1 h and the reaction was stopped as indicated in the Methods. Rat (●) 0.05 mg, monkey (■) 0.076 mg, sheep (○) 0.24 mg and chicken (□) 0.076 mg.

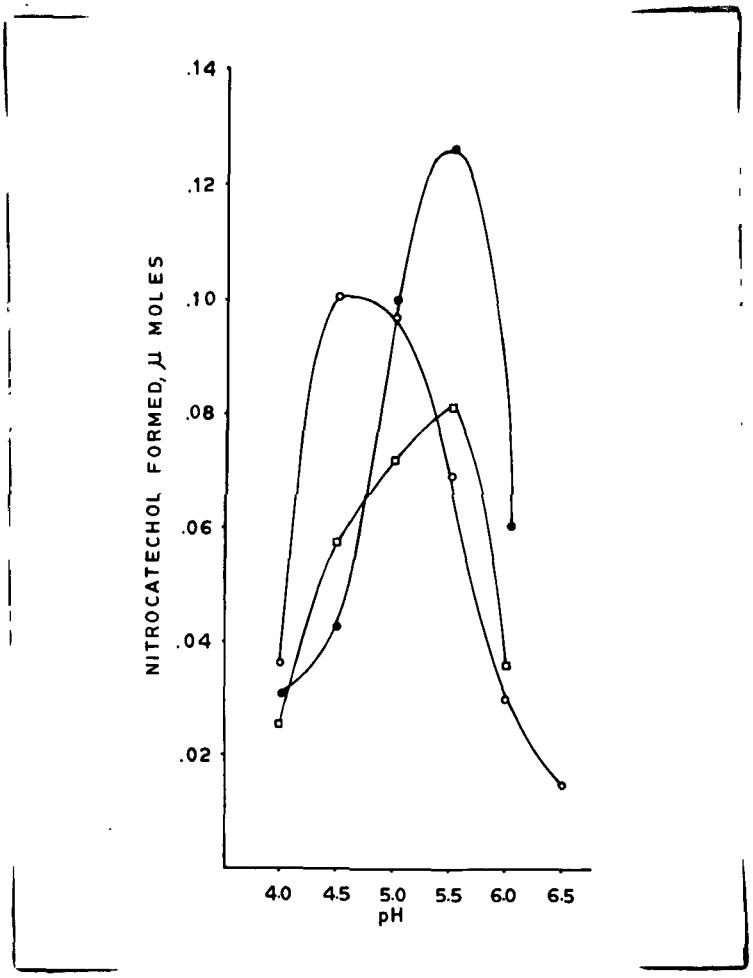

FIG.4. pH activity curves of rat, monkey and sheep arylsulphatase B. The incubation mixture consisted of 50 μmol of sodium acetate buffer of various pH values, 5 μmol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction I) in a total volume of 0.3 ml. The reaction mixture was incubated at 37°C for 1 h and the reaction was stopped as indicated in the Methods. Enzyme protein used: Rat (●) 0.04 mg, monkey (□) 0.3 mg, and sheep (○) 0.32 mg.

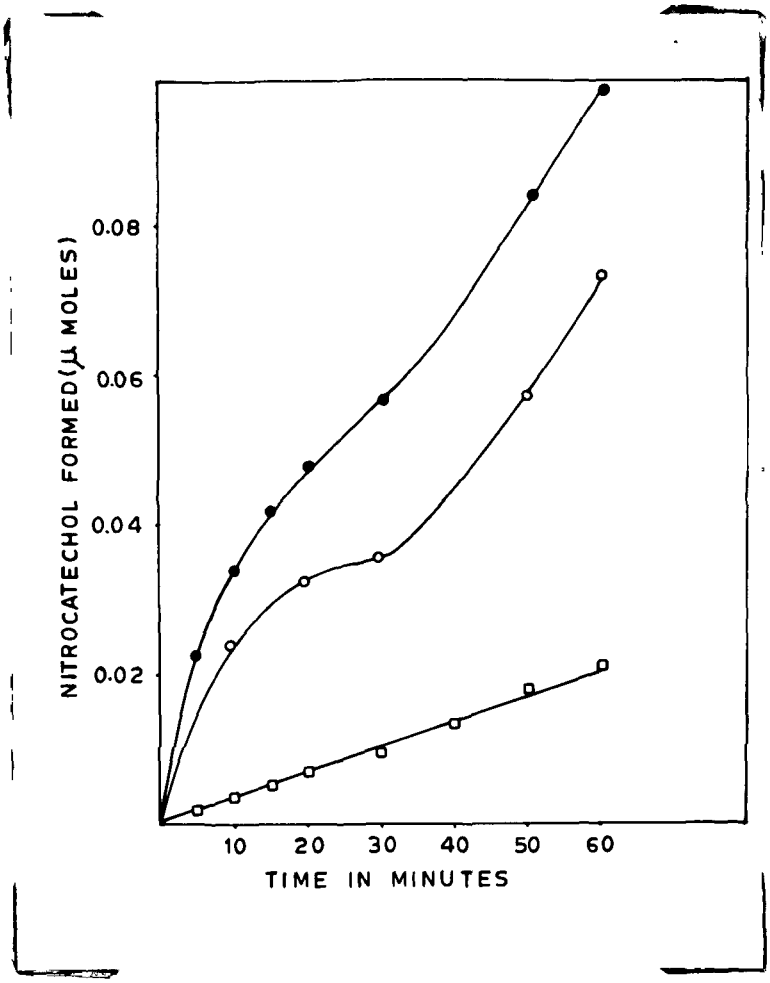

FIG. 5. Time activity curves of rat brain arylsulphatase A at different protein concentrations. The incubation mixture consisted of 50 μmol of sodium acetate buffer pH 5.0, 1 μmol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction II) in a total volume of 0.3 ml. The reaction mixture was incubated at 37°C and the reaction was stopped as indicated in the Methods. Enzyme protein used: 0.05 mg (O), 0.1 mg (●), and 0.05 mg and 0.1 mM sodium pyrophosphate (□).

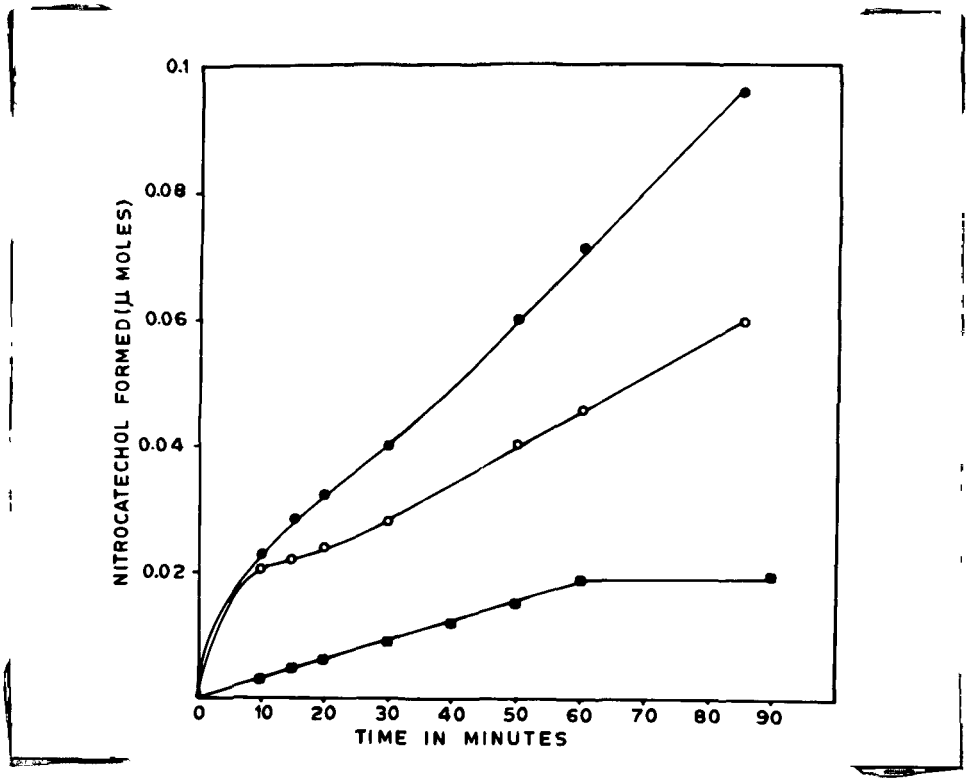

FIG.6. Time activity curves for monkey brain arylsulphatase A at different protein concentrations. The incubation mixture consisted of 50 μmol of sodium acetate buffer pH 4.5, 1 μmol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction II) in a total volume of 0.3 ml. The reaction mixture was incubated at 37°C and the reaction was stopped as indicated in the Methods. Enzyme protein used: 0.038 mg (O), 0.076 mg (●) and 0.076 mg and 2.5×10^{-4}M sodium pyrophosphate (■).

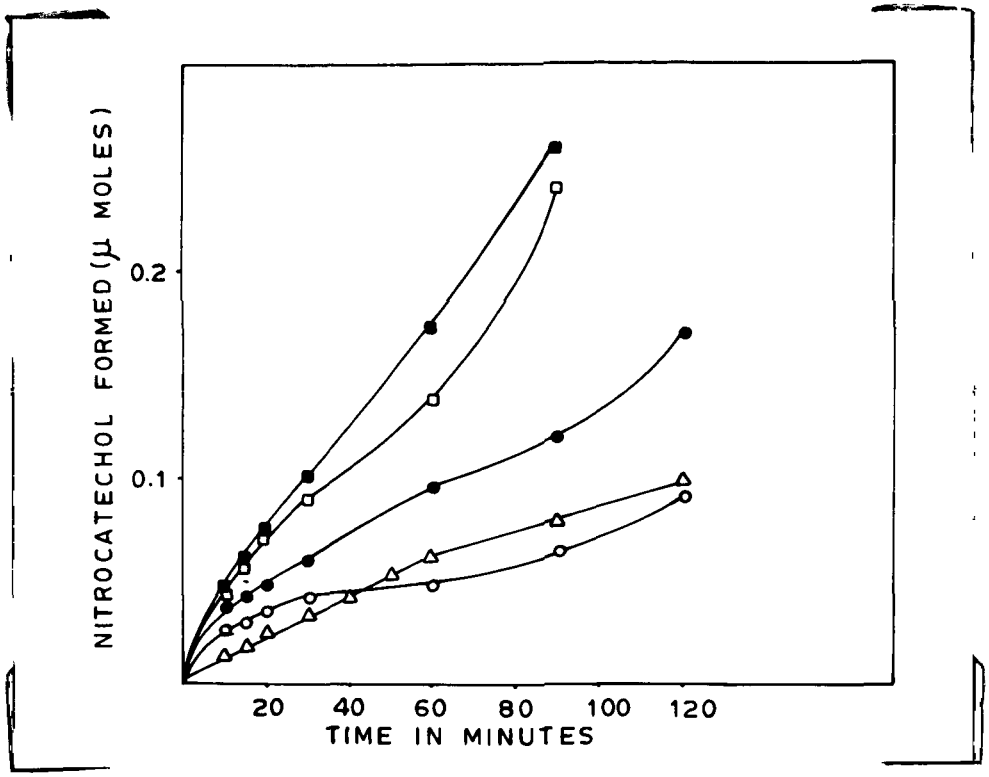

FIG.7. Time activity curves for sheep brain arylsulphatase A at different protein concentrations. The incubation mixture consisted of 50 μmol of sodium acetate buffer pH 5.5, 1 μmol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction II) in a total volume of 0.3 ml. The reaction mixture was incubated at 37°C and the reaction was stopped as indicated in the Methods. Enzyme protein used: 0.095 mg (○), 0.19 mg (●), 0.304 mg (□), 0.38 mg (■) and 0.304 mg and 2.5×10^{-4}M sodium pyrophosphate (△).

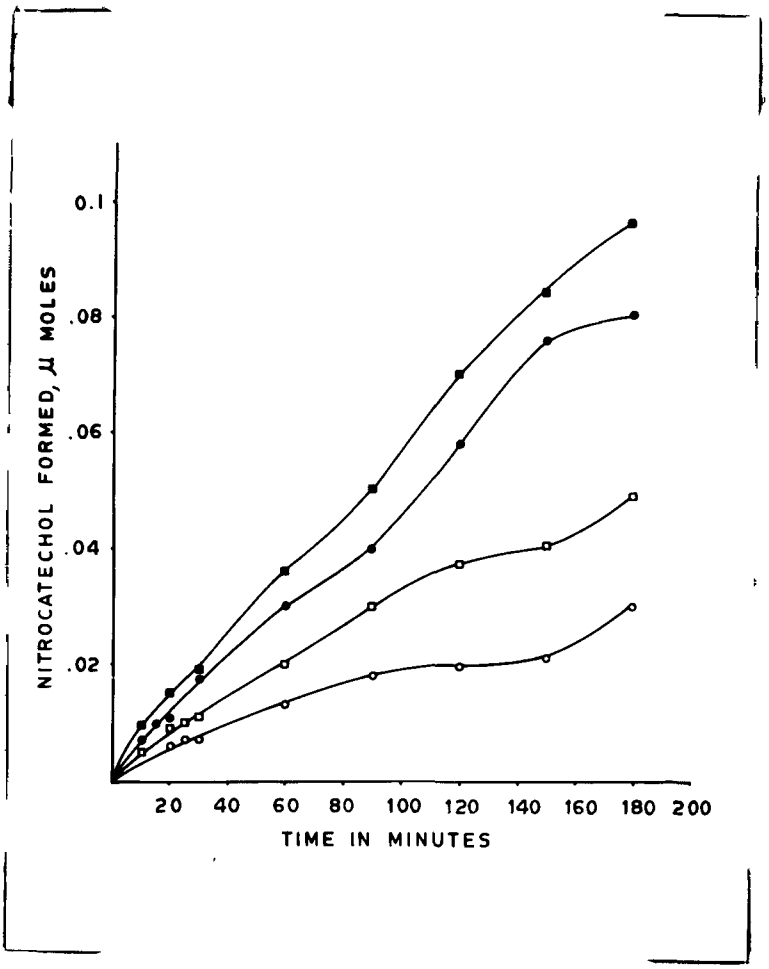

FIG.8. Time-activity curves of chicken brain arylsulphatase A at different protein concentrations. The incubation mixture consisted of 50 μ mol of acetate buffer pH 4.5. 1 umol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction I) in a total volume of 0.3 ml. The tubes were incubated at 37°C and the reaction was stopped as indicated in the Methods. Enzyme protein used: 0.095 mg (O), 0.19 mg (□), 0.304 mg (●) and 0.38 mg (■).

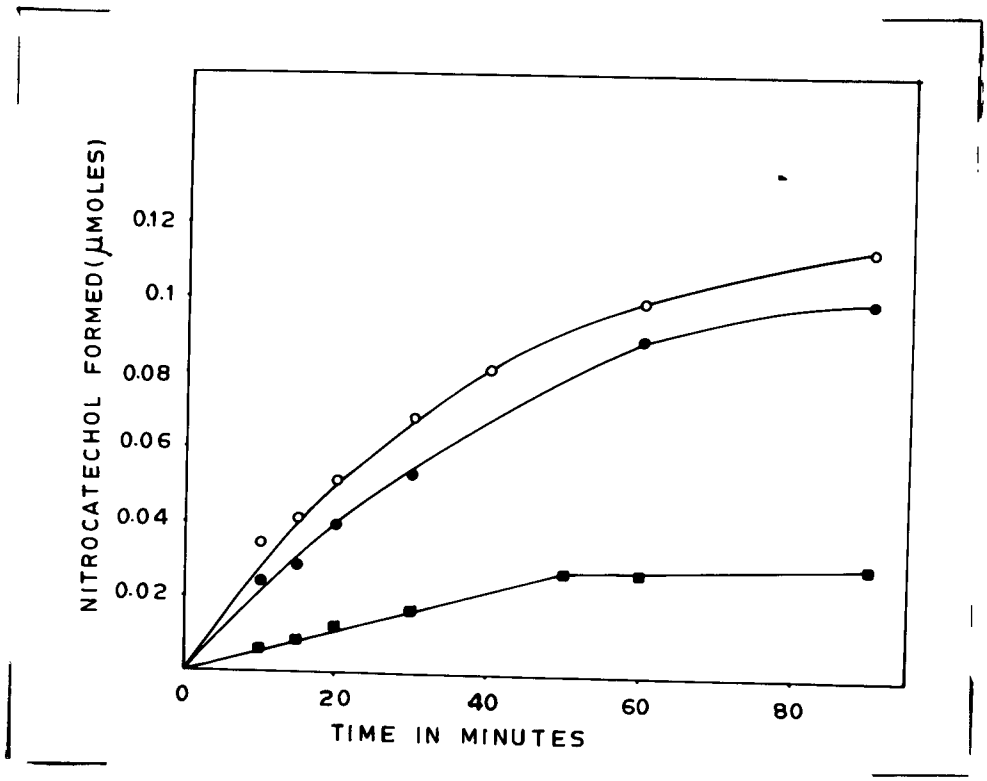

FIG.9. Time activity curves of arylsulphatase B of various animal species. The incubation mixture consisted of 50 μmol of sodium acetate buffer with appropriate pH value for each animal species, 5 μmol of p-nitrocatechol sulphate and enzyme (zinc acetate fraction I) in a total volume of 0.3 ml. The tubes were incubated at 37°C and the reaction was terminated as indicated in the Methods. Enzyme protein used: Rat (●) 0.04 mg, monkey (■) 0.2 mg, and sheep (O) 0.32 mg.

relationship in the presence of sodium pyrophosphate as reported by Baum and Dodgson (32). Arylsulphatase B of rat, monkey and sheep exhibits normal time-activity relationship (Fig. 9).

Km value and effect of sulphite and phosphate ions: Table VI compares the Km values of arylsulphatases A and B in various animal species. The double reciprocal curves for arylsulphatases A and B of various animal species are shown in Fig. 10 and 11. The effect of sulphite and phosphate ions was studied on sheep and chicken brain arylsulphatase A (Fig. 12 and 13). The Ki values for sheep and chicken brain enzymes using sulphite ions as inhibitor are 0.7 X 10^{-5}M and 0.8 X 10^{-5}M respectively. The Ki values for phosphate ions in sheep and chicken brain are 2.57 X 10^{-4}M and 0.8 X 10^{-4}M. The reported values of Ki for human brain arylsulphatase A for sulphite and phosphate ions are 1.98 X 10^{-6}M and 3.5 X 10^{-5}M respectively (34).

As mentioned above, the gel electrophoresis of zinc acetate fraction I and II results in the complete separation of arylsulphatases A and B from each other. The kinetics of monkey brain arylsulphatases A and B eluted from the gel were also studied. It was found that the

CHAPTER II

TABLE VI

COMPARISON OF Km VALUES OF ARYLSULPHATASE A AND B FROM VARIOUS ANIMAL SPECIES

Animal species	Km Value	
	Arylsulphatase A	Arylsulphatase B
Rat	1.8×10^{-3} M	4.30×10^{-3} M
Monkey	1.47×10^{-3} M	3.70×10^{-3} M
Sheep	4.5×10^{-3} M	9.09×10^{-3} M
Chicken	3.1×10^{-3} M	-
Human*	1.5×10^{-3} M	-

*For comparison, this value was taken from Balasubramanian and Bachhawat (Ref. No.34).

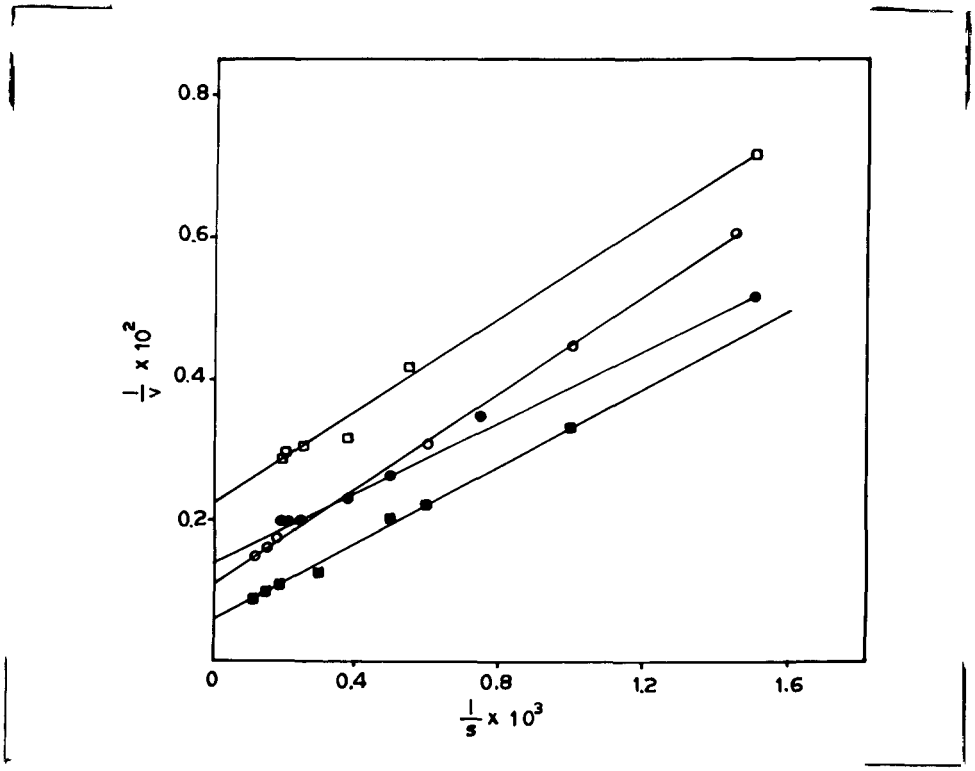

FIG.10. The double reciprocal plots of arylsulphatase A of various animal species. The incubation mixture consisted of 50 µmol of sodium acetate with appropriate pH for each animal species, different concentrations of p-nitrocatechol sulphate, and enzyme in a total volume of 0.3 ml. The tubes were incubated at 37°C for 60 min. and the reaction was terminated as indicated in the Methods. Enzyme protein used: Rat (●) 0.05 mg, monkey (□) 0.108 mg, sheep (■) 0.24 mg and chicken (○) 0.345 mg.

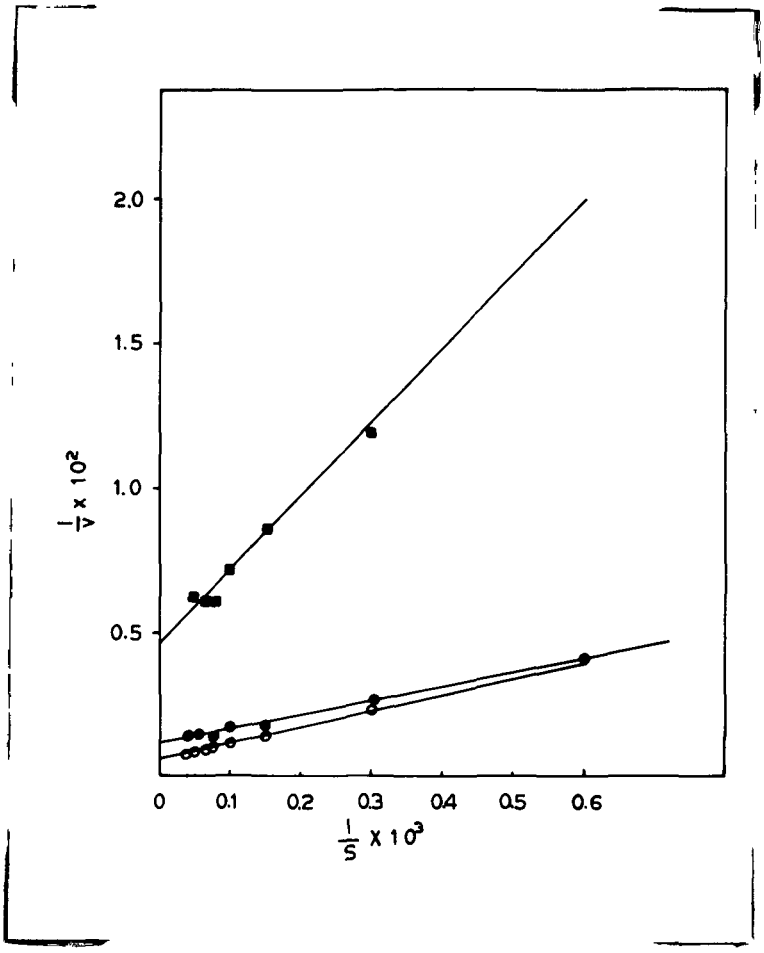

FIG.11. The double reciprocal plots of aryl-sulphatase B of various animal species. The incubation mixture consisted of 50 µmol of sodium acetate buffer with appropriate pH value for each animal species, different concentrations of p-nitrocatechol sulphate and enzyme in a total volume of 0.3 ml. The tubes were incubated at 37°C for 60 min. and the reaction was terminated as indicated in the Methods. Enzyme protein used: Rat (●) 0.042 mg, monkey (■) 0.28 mg, and sheep (O) 0.32 mg.

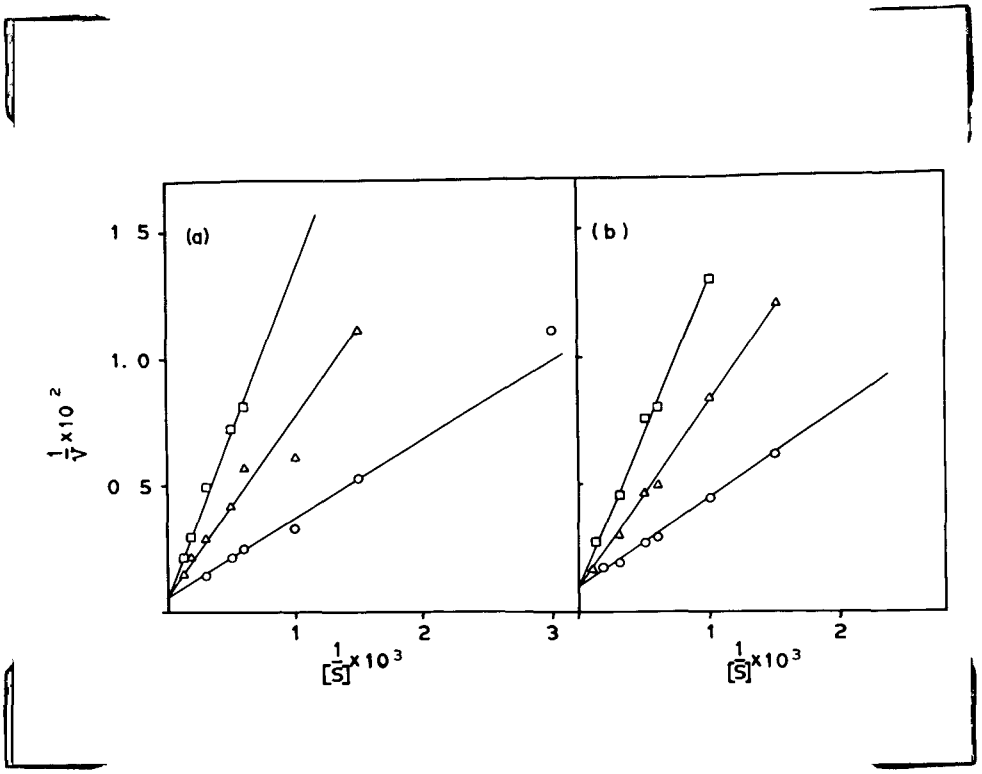

FIG.12. Lineweaver-Burk plots showing the effect of sodium sulphite on sheep (a) and chicken (b) brain arylsulphatase A. (a) The assay mixture consisted of 50 µmol of sodium acetate buffer pH 5.5, different concentrations of p-nitrocatechol sulphate and 0.24 mg enzyme protein (zinc acetate fraction II) in a total volume of 0.3 ml. (b) The reaction mixture consisted of 50 µmol of sodium acetate buffer pH 4.5, different concentrations of p-nitrocatechol sulphate, 0.345 mg enzyme protein (zinc acetate fraction I) in a total volume of 0.3 ml. The tubes were incubated at 37°C for 60 min. and nitrocatechol formed was estimated as indicated in the Methods. Sodium sulphite concentration ○, None; △, 0.5 X 10^{-5}M, and □, 2.5 X 10^{-5}M.

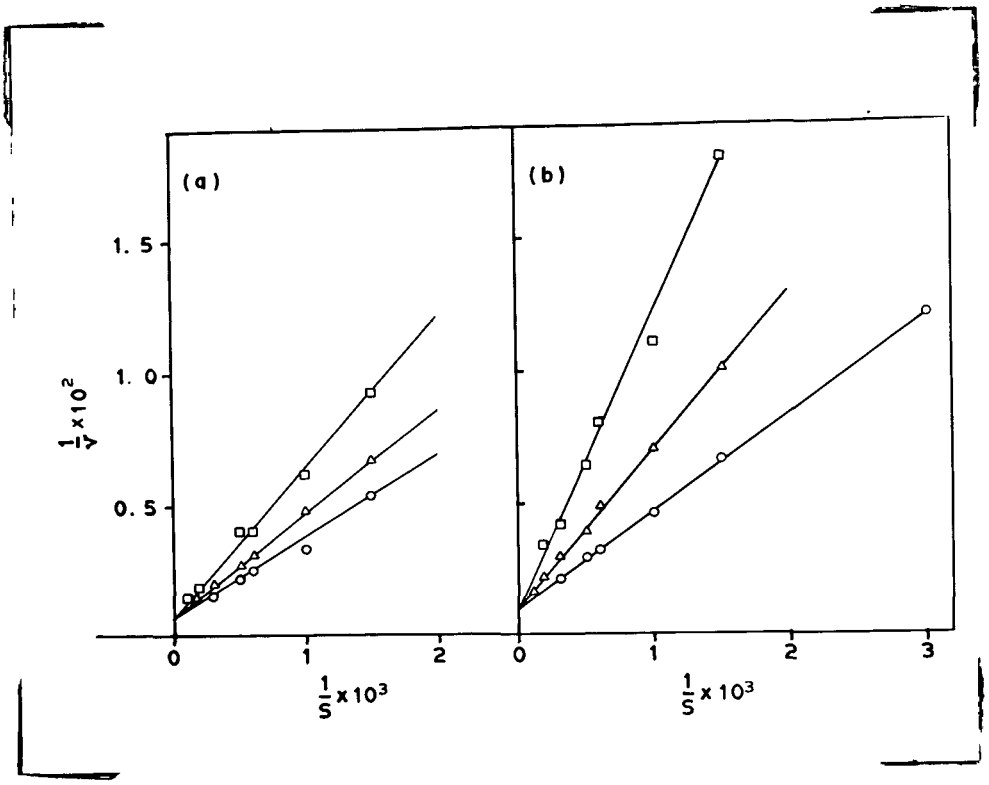

FIG.13. Lineweaver-Burk plots showing the effect of potassium phosphate on sheep (a) and chicken (b) brain arylsulphatase A. (a) The assay mixture consisted of 50 µmol of sodium acetate buffer pH 5.5, different concentrations of p-nitrocatechol sulphate, 0.24 mg enzyme protein (zinc acetate fraction II) in a total volume of 0.3 ml. (b) The assay mixture consisted of 50 µmol of sodium acetate buffer pH 4.5, different concentrations of p-nitrocatechol sulphate and 0.345 mg enzyme protein (zinc acetate fraction I) in a total volume of 0.3 ml. The tubes were incubated at 37°C for 60 min. and nitrocatechol formed was estimated as indicated in the Methods. Potassium phosphate concentration ○, none; △, 0.5×10^{-4}M; and □, 2.5×10^{-4}M.

pH optimum and Km value of the arylsulphatases A and B were the same before and after electrophoresis (Table VII). This indicates that even though the arylsulphatases A and B from the zinc acetate fraction I and II were contaminated with each other to a miror extent, it did not significantly affect the pH optimum and Km value.

5. Discussion:

The results reported in this Chapter on arylsulphatase A and B activity are based upon the differential assay procedure of Baum, Dodgson and Spencer (99). The results indicate that this differential assay method may be used if proper substrate concentration was employed. The assay conditions used were, optimal for arylsulphatases A and B of chicken, monkey, human and rat whereas the optimal conditions for pigeon, frog and rabbit were not determined. In the case of sheep brain arylsulphatases A and B, the substrate concentration employed was not optimal.

The results of comparative studies on the activity of arylsulphatases A and B in different species show that the proportions of these enzymes vary from one species to another. It is interesting to note that there is a relationship between the mucopolysaccharide

CHAPTER II

TABLE VII

COMPARISON OF pH AND Km VALUES OF ARYLSULPHATASE A AND B OF MONKEY BRAIN

	Arylsulphatase A		Arylsulphatase B	
	pH value	Km value	pH value	Km value
Before electrophoresis	4.5	1.47×10^{-3} M	5.5	5.7×10^{-3} M
After electrophoresis	4.5	1.44×10^{-3} M	5.5	5.7×10^{-3} M

content and the amount of arylsulphatase B. Thus, in rat, monkey and man, where the mucopolysaccharide contents are very high (110) arylsulphatase B accounts for the greater part of the total arylsulphatase activity on the other hand in birds and sheep where the mucopolysaccharide contents are low and cerebroside 3-sulphate concentration is high compared to that in rat and man (111) arylsulphatase A is present in higher amount.

The behaviour of chicken brain arylsulphatase A under zinc acetate fractionation and acrylamide gel-electrophoresis suggests that it is similar to arylsulphatase B of other species studied as far as the charge on the proteir molecule is concerned. The other properties such as Km value and the inhibitory effect of phosphate and sulphite ions, suggest that this arylsulphatase is similar to sheep brain arylsulphatase A and human brain arylsulphatase A (34). The time-activity relationship of chicken brain arylsulphatase A shows the same abnormality as seen in all the other species studied except that it is less pronounced.

The regional distribution studies on monkey brain show that arylsulphatase A and B activities are present in all regions. It may be noted that activity of arylsulphatase A is always high in white matter which is

the richest source of cerebroside 3-sulphates (112).

The investigations on the activities of arylsulphatases A and B in developing rat brain indicate a relationship between mucopolysaccharides content and activity of arylsulphatase B. Singh and Bachhawat (113) have reported that the mucopolysaccharide content of rat brain decreases rapidly and reaches a minimum on the 5th day after birth; further increase in age results in a sharp peak on 7th day followed by a plateau between 11 and 19 days, after which there is a gradual decrease in mucopolysaccharide content. The activity of arylsulphatase B also increases after birth, reaches a peak on 5th day and then falls off. As age increases the activity of arylsulphatase B becomes more or less constant after another peak of activity on 20th day which is the time when mucopolysaccharides start decreasing.

Austin et al (103) have reported that arylsulphatase A levels tend to parallel the level of incorporation of (^{35}S)-sulphate into cerebroside 3-sulphates of rat brain and the levels of arylsulphatase A and cerebroside 3-sulphate reach their peak in young rat brain during myelination period. Our results on the

activity of arylsulphatase A in developing rat brain show that the activity is high in the 18 to 25 days old rat. Further, Hauser (114) has reported that the cerebroside 3-sulphate content of rat brain is high in the same age groups. These observations lend additional support to the idea that there is a relationship between arylsulphatase A activity and cerebroside 3-sulphate content of brain.

6. Summary:

The relative proportions of arylsulphatases A and B were determined by the method of Baum, Dodgson and Spencer (99) in brains of various animal species and it was found that there was a considerable variation in the concentration of these two enzymes. Arylsulphatases A and B of various animal species including rat, man, monkey, sheep and chicken were partially separated using zinc acetate fractionation procedure and gel-electrophoresis. The chicken brain arylsulphatase A had a similar electrophoretic mobility to that of arylsulphatase B of other species. Further, chicken brain arylsulphatase A precipitated at a zinc acetate concentration of α 0.005M, a condition under which arylsulphatase B from the brain of other species

precipitated. The kinetic properties such as Km value and inhibitory effect of sulphite and phosphate ions indicated that chicken brain arylsulphatase A was similar to arylsulphatase A of other species. The results on regional distribution of arylsulphatase A and B activities in monkey brain and in developing rat brain suggest a relationship between arylsulphatase A and cerebroside 3-sulphates and arylsulphatase B and mucopolysaccharides.

CHAPTER III.

PURIFICATION AND PROPERTIES OF ARYLSULPHATASE A FROM CHICKEN BRAIN

CHAPTER III.

PURIFICATION AND PROPERTIES OF ARYLSULPHATASE A FROM CHICKEN BRAIN

1. Introduction:

Lysosomal arylsulphatases A and B have been found in all mammalian tissues investigated. The two enzymes are distinguished by different pH optima, substrate affinities, anomalous kinetics and electrophoretic mobilities. Arylsulphatases A and B had been extensively purified from various mammalian tissues (35, 55, 68, 71, 74, 91). Ignorance about the natural substrate made it difficult to assign any physiological role to these enzymes. A significant decrease in arylsulphatase A activity in the genetic disorder metachromatic leukodystrophy, which is characterised by abnormal accumulation of cerebroside 3-sulphate, was observed by Austin et al (65,81). Mehl and Jatzkewitz (79) have reported that arylsulphatase A from pig kidney in the presence of a heat-stable factor can degrade cerebroside 3-sulphate. Chapter II has indicated the differences in proportions and physicochemical properties of brain arylsulphatases in various animal species. Chicken brain arylsulphatase resembles arylsulphatase A of other species in its kinetic properties such as Km value,

anomalous time-activity relationship and inhibitory effect of sulphite, phosphate and sulphate ions. However, its electrophoretic mobility and behaviour under zinc acetate fractionation is similar to the arylsulphatase B of other species.

This chapter describes the purification and properties of arylsulphatase A from chicken brain. Some properties of this partially purified arylsulphatase A are also compared with those of the human brain arylsulphatase A.

2. Materials:

Dipotassium salt of p-nitrocatechol sulphate, phenolphthalein β-glucuronide, cytochrome C, myoglobin, haemoglobin, bovine serum albumin, and γ-globulin were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). p-Nitrophenyl β-galactoside was purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Sephadex G-75, Sephadex G-200, DEAE-Sephadex A-50 and Blue dextran were purchased from Pharmacia, Uppsala, Sweden. (^{35}S) Sulphate was obtained from Atomic Energy Establishment, Trombay, India, and all chemicals used were of analytical grade.

3. Methods:

3.(i) Assay of chicken brain arylsulphatase A:

The enzyme was assayed by measuring the production of nitrocatechol from p-nitrocatechol sulphate. The assay system consisted of 0.2M-sodium acetate buffer, pH 5.0, 6mM-nitrocatechol sulphate and enzyme in a total volume of 0.1 ml. The incubation was done at 37°C for 20 min., then the reaction was stopped by addition of 2.9 ml of 0.11M-sodium hydroxide. The nitrocatechol that formed was measured in a Klett-Summerson Colorimeter by using the 50 filter. The amount of nitrocatechol formed was calculated from a standard curve.

3.(ii) Preparation of adenosine 3'-phosphate 5'-(^{35}S) sulphato-phosphate:

The active sulphate synthesizing enzyme was prepared according to the method of Panikkar and Bachhawat (115) using rat liver instead of sheep liver.

The liver was homogenised in 4 ml/g of 1.15% isotonic KCl and the homogenate was centrifuged at 12,000 X g for 90 min. The supernatant was subjected to ammonium sulphate precipitation using 3.75M-ammonium sulphate solution. The supernatant solution obtained after 1.5M-ammonium sulphate precipitation was again

raised to 2.1M-ammonium sulphate concentration and centrifuged. The precipitated protein which contained the sulphate activating enzyme was dissolved in a minimum volume of 0.02M-tris-HCl pH 7.4. The solution was desalted by passing through Sephadex G-75 column (2.2 cm X 22 cm) previously equilibrated with 0.02M-tris-HCl pH 7.4. The enzyme was eluted with the same buffer in the void volume. This was used as enzyme for the preparation of adenosine 3'-phosphate 5'-sulphato-phosphate without any further treatment.

The reaction mixture consisted of 400 μmoles of tris-HCl buffer pH 8.0, 20 μmoles of magnesium chloride, carrier-free (^{35}S) sulphate (600 μci), 30 μmoles of ATP, 20 μmoles of freshly neutralised cysteine-hydrochloride and the liver enzyme in a total volume of 3.0 ml. After incubation for 60 min. at 37°C the reaction was stopped by placing the tubes in a boiling water-bath for 30 sec. and the reaction mixture chilled in ice.

The precipitated protein removed by centrifugation and adenosine 3'-phosphate 5'-(^{35}S) sulphato-phosphate in the supernatant was separated from other nucleotides and inorganic sulphate by descending paper chromatography on Whatman No.3 MM paper using solvent system

ethanol:1M-ammonium acetate solution (7.5:3) pH 7.2 for 24 h (116). After counting the paper strips for radioactivity adenosine 3'-phosphate 5'-(^{35}S) sulphatophosphate was eluted from the paper with water at 4°C, concentrated at 36°C in vacuum and stored at -18°C.

3.(iii) Measurements of radioactivity:

Radioactivity of paper strips was determined in a Packard Tri-Carb liquid scintillation counter using 1.0 ml of scintillation mix.—— Toluene-PPO (2,5 diphenyloxazole) - POPOP (1,4-bis-2(5-phenyloxazolyl) benzene)-naphthalene mix. (Toluene 100 ml PPO 1.0 g., POPOP 0.05 g. and naphthalene 20 g.).

3.(iv) β-Glucuronidase:

The enzyme was assayed by the method of Plapp &Cole (117) with phenolphthalein β-glucuronide as substrate.

3.(v) β-Galactosidase:

The enzyme was assayed by the method Gatt & Rapport (118) with p-nitrophenyl β-galactoside as substrate.

3.(vi) Acid phosphatase:

The enzyme was assayed by the method described

by Austin et al (81).

3.(vii) Inorganic pyrophosphatase:

The enzyme activity was determined by the method of Sussman & Laga (119) with inorganic pyrophosphate as substrate and the P_i released was measured by the method of Fiske and Subba Row (120).

3.(viii) Adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase:

The enzyme was assayed by the method of Balasubramanian and Bachhawat (121), with adenosine 3'-phosphate 5'-(^{35}S) sulphatophosphate.

3.(ix) Polyacrylamide gel-electrophoresis:

This was carried out as described in Chapter II.3.(iv) in 0.03M-sodium barbitone buffer, pH 8.0 for 15 h at 8 mA/tube in a Buchler instrument. Enzyme was eluted from the gel as described in Chapter II.3.(iv). The proteins were stained by Coomassie Brilliant Blue (108).

3.(x) Molecular weight determination:

The molecular weight of chicken brain arylsulphatase A was determined by the method of Andrews (122) using sephadex G-200. Sephadex G-200 particle size 40-120 μ was allowed to swell for 5 days in 0.1M-KCl.

Columns (2.4 cm X 50 cm, bed volume - 226 ml) with satisfactory flow rates (15-18 ml/h) were packed in cold (4-6°C) with the swollen gel, previously deaerated under reduced pressure. The columns were equilibrated with 0.05M-tris-HCl buffer, pH 7.5, containing 0.1M-KCl. Samples were dissolved in equilibration solution (2 ml) and applied to the top of the columns. The effluent was collected in 2 ml fractions with a fraction collector with siphon. The columns were calibrated by the use of cytochrome C, myoglobin, haemoglobin, bovine serum albumin and γ-globulin. The void volume (70 ml) of the columns was determined by using the blue dextran.

3.(xi) Preparation of enzyme:

All operations were carried out at 4°C unless otherwise mentioned.

Frozen chicken brain (500 g) was homogenised with 1 litre of 0.2M-sodium acetate buffer, pH 5.0, in a Waring blender for 2 min. The homogenate (1700 ml) was centrifuged at 12,000 g for 30 min. and supernatant (750 ml) adjusted to 1.64M by adding 4.1M-ammonium sulphate. The precipitate that formed was dissolved in 0.02M-sodium acetate buffer, pH 5.0, and dialysed for 8 h against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4.

After dialysis the solution was stored overnight at 0°C and insoluble material was removed by centrifugation at 12,000 g for 30 min. The supernatant (62 ml) was subjected to ethanol precipitation. Aldehyde-free ethanol was added dropwise (1 ml/min) with constant mechanical stirring to a final concentration of 40% (v/v). After 10 min. the solution was centrifuged at 12,000 g for 30 min. The precipitate was discarded and the supernatant was dialysed against 100 vol. of 0.02M-tris-HCl buffer, pH 7.4, for 8 h. After dialysis the solution (180 ml) was adjusted to 40% saturation by addition of solid ammonium sulphate. The precipitate obtained after centrifugation for 30 min. at 12,000 g was dissolved in 0.02M-sodium acetate buffer, pH 5.0, and was dialysed overnight against 100 vol. of 0.02M-tris HCl buffer, pH 7.4.

The dialysed preparation (8 ml) was applied to a Sephadex G-200 column (1.28 cm X 38 cm) equilibrated with 0.02M-tris-HCl buffer, pH 7.4, and eluted in 5 ml fractions with the same buffer at a flow rate of 15 ml/h. The fractions that contained the enzyme were pooled and made 90% saturated with ammonium sulphate. The precipitate that was obtained after centrifugation was dissolved in the minimum amount of 0.02M-sodium acetate

buffer, pH 5.0, and was dialysed overnight against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4.

The clear solution (7 ml) was then applied to a column (0.5 cm X 7 cm) of DEAE-Sephadex A-50 equilibrated with 0.02M-tris-HCl buffer, pH 7.4. The column was washed with this buffer at a flow rate of 12 ml/h. The washings did not contain any enzyme activity and were discarded. Stepwise elution was done with gradient of 0.05-0.1M-NaCl in 0.02M-tris-HCl buffer, pH 7.4. Most of the enzyme activity was eluted in the 0.1M-NaCl fraction. This fraction was used to study the enzyme properties.

3.(xii) Purification of arylsulphatase A from human brain:

Arylsulphatase A of human brain was purified by the method of Balasubramanian and Pachhawat (34).

3.(xiii) Protein determination:

Protein was determined as described in Chapter II.3.(v).

4. Results:

Chicken brain arylsulphatase A was purified 2000-fold (Table 1) with an overall recovery of 14% by

CHAPTER III

TABLE I

PURIFICATION OF ARYLSULPHATASE A FROM CHICKEN BRAIN

Enzyme fraction	Total volume (ml)	Total protein (mg)	Total enzyme units*	Specific activity	Recovery (%)
Homogenate	1700	54400	316	0.015	100
12000 g supernatant	750	3750	468	0.120	57
1.64M-Ammonium sulphate	62	446	1023	2.300	125
Ethanol supernatant	180	300	518	1.700	63
40% Ammonium sulphate	16	198	614	3.100	72
G-200 Gel filtration	7	36	403	11.200	49
DEAE-Sephadex fraction	32	3.84	115	30.000	14

The homogenate and 12000 g supernatant were assayed after 3 h dialysis against 0.02 M-tris-HCl buffer pH 7.4.

*One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of nitrocatechol from p-nitrocatechol sulphate in 20 min under usual assay conditions.

the above procedure. There was a considerable increase in total enzyme activity when the 12,000 g supernatant was fractionated with 1.64M-ammonium sulphate. This might be caused by the removal of an endogenous inhibitor, because when 12,000 g supernatant that had been dialysed was added to the purified enzyme, there was about 40% inhibition of enzyme activity. For a reproducible result with ethanol fractionation it was necessary to maintain the temperature at 4-6^{o}C.

The elution profile of chicken brain arylsulphatase A through a Sephadex G-200 column is shown in Fig. 1. The enzyme was eluted from the column as a single symmetrical peak, which did not coincide with protein peak. Fig. 2 shows the elution profile of the same enzyme through a DEAE-Sephadex A-50 column. Most of the enzyme activity was eluted in the 0.1M-NaCl fraction as a single peak. The purified enzyme preparation was completely free from B-glucuronidase, B-galactosidase, acid phosphatase, inorganic pyrophosphatase and adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase activity and could be stored for 2 months at 0^{o}C without any appreciable loss of enzyme activity.

The purity of this chicken brain arylsulphatase A as judged by polyacrylamide gel-electrophoresis (Fig.3)

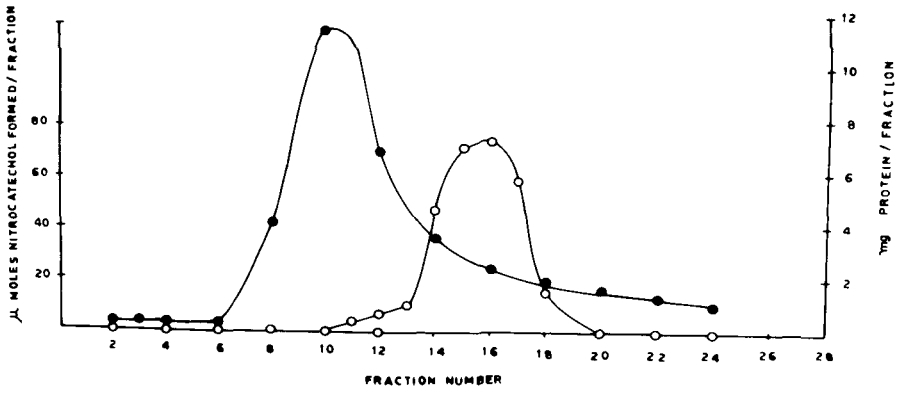

FIG.1. Sephadex G-200 elution profile of chicken brain arylsulphatase A. 5 ml fractions were collected. The experimental details and analysis of fractions for the protein and enzyme activity are described in the Methods. ●, Protein; ○, arylsulphatase A activity.

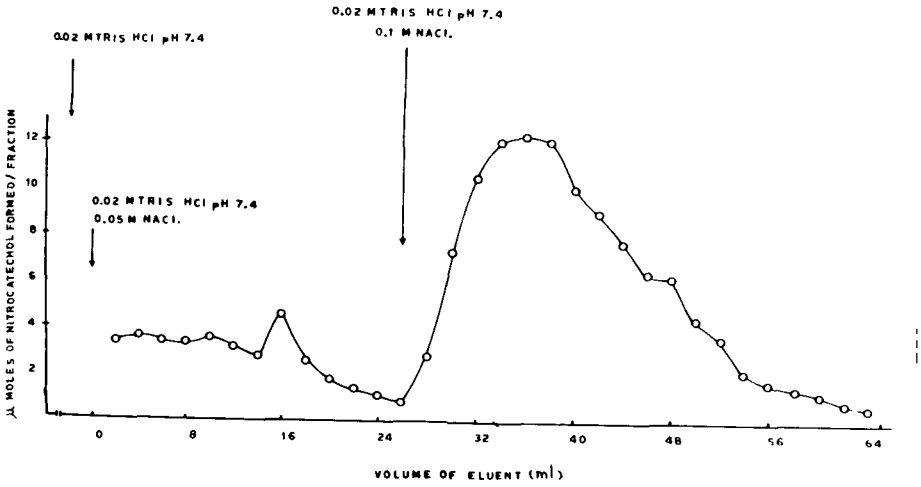

FIG.2. DEAE-Sephadex A-50 elution profile of chicken brain arylsulphatase A. 2.0 ml fractions were collected and analysed for enzyme activity as described in the Methods.

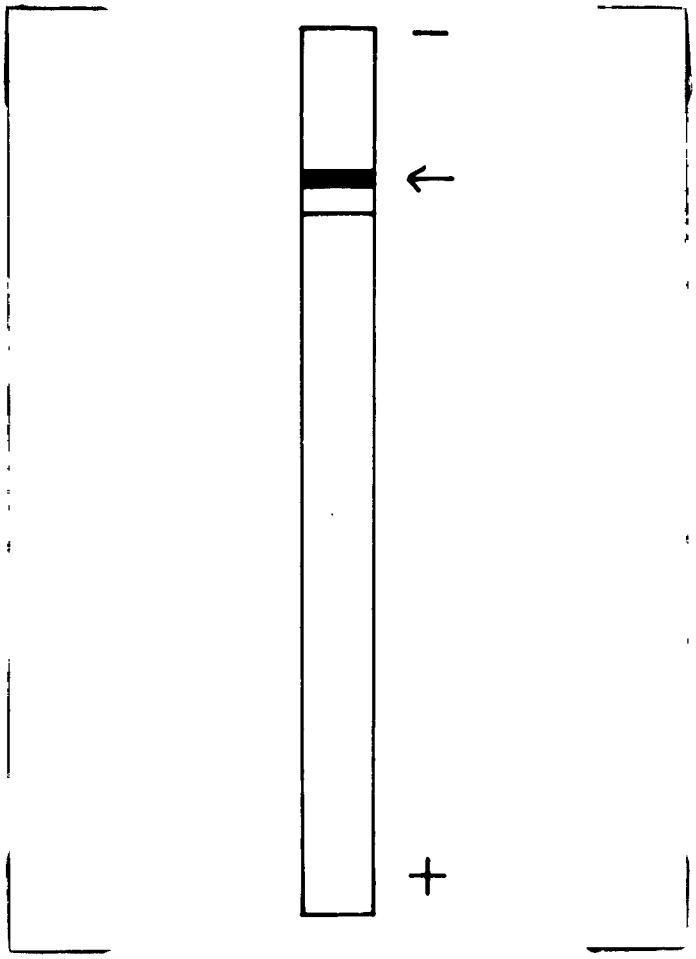

FIG.3. Electrophoretic mobility of chicken brain arylsulphatase A. A 7.5% polyacrylamide gel in a 17 cm X 1 cm tube was used. The conditions for electrophoresis were as described in the Methods. The major protein band, indicated by arrow, contained the enzyme activity.

indicated that the preparation was not homogeneous. It had two protein bands. The arylsulphatase activity in various segments of the gel was assayed after being eluted and the activity was located in the major protein band, whereas the minor protein band had no enzyme activity. In Chapter II.4.(i) it is described that the electrophoretic mobility of chicken brain arylsulphatase A was similar to that of arylsulphatase B of other animal species. The electrophoretic mobility of purified preparation was the same (3 cm) as reported in Chapter II.4(i).

4.(i) Properties of chicken brain arylsulphatase A:

pH optimum: The enzyme had a pH optimum at 5.5 in sodium acetate buffer (Fig.4) when incubated for 5 min. but when the time of incubation was increased to 30 min. the pH optima shifted from 5.5 to 5.0 as shown in Fig.5. Baum, Dodgson and Spencer (20) reported that after incubation for 10 min. the human liver arylsulphatase A showed optimum activity at two pH values, which over long incubation periods or at higher enzyme concentration merged into a single pH optimum. Harinath and Robins (35) found only one pH optimum for the human brain arylsulphatase A with 4-methylumbelliferone sulphate as a substrate. Phosphate and sulphite ions did not shift the pH optimum

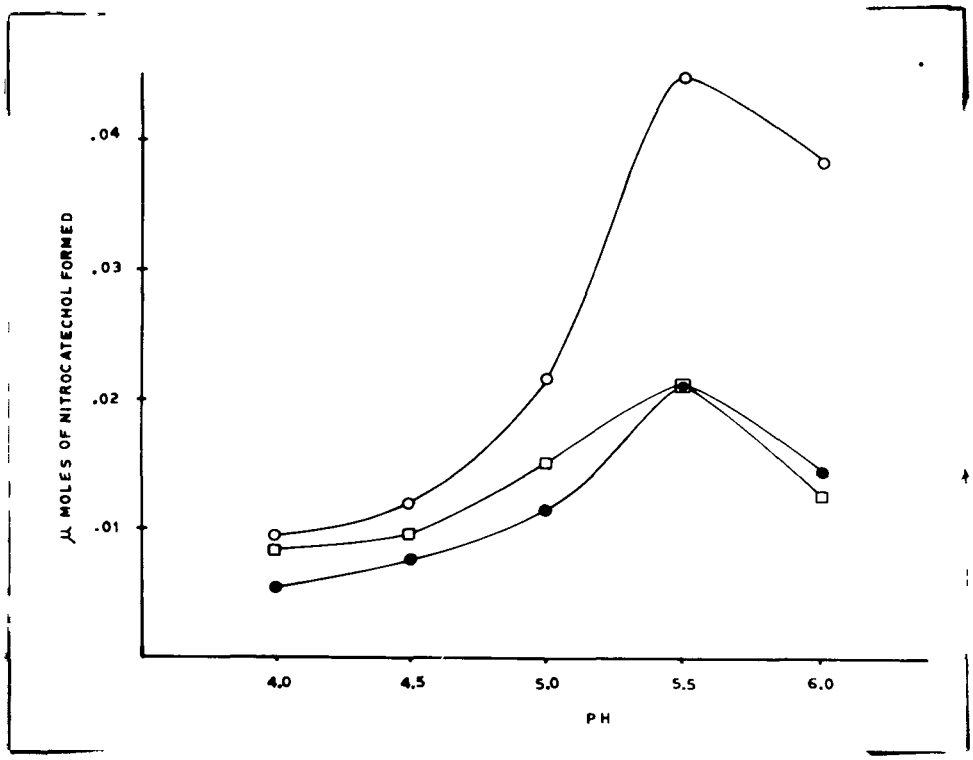

FIG.4. pH activity curve of chicken brain arylsulphatase A with phosphate and sulphite ions. The assay mixture consisted of 0.2M-sodium acetate buffer of various pH values, 6mM p-nitrocatechol sulphate and 4.8 μg of enzyme protein in a total volume of 0.1 ml. After incubation for 5 min. the reaction was terminated and nitrocatechol formed was determined as described in the Methods.
○, No addition; □, 0.25mM-phosphate; ●, 25 μM-sulphite.

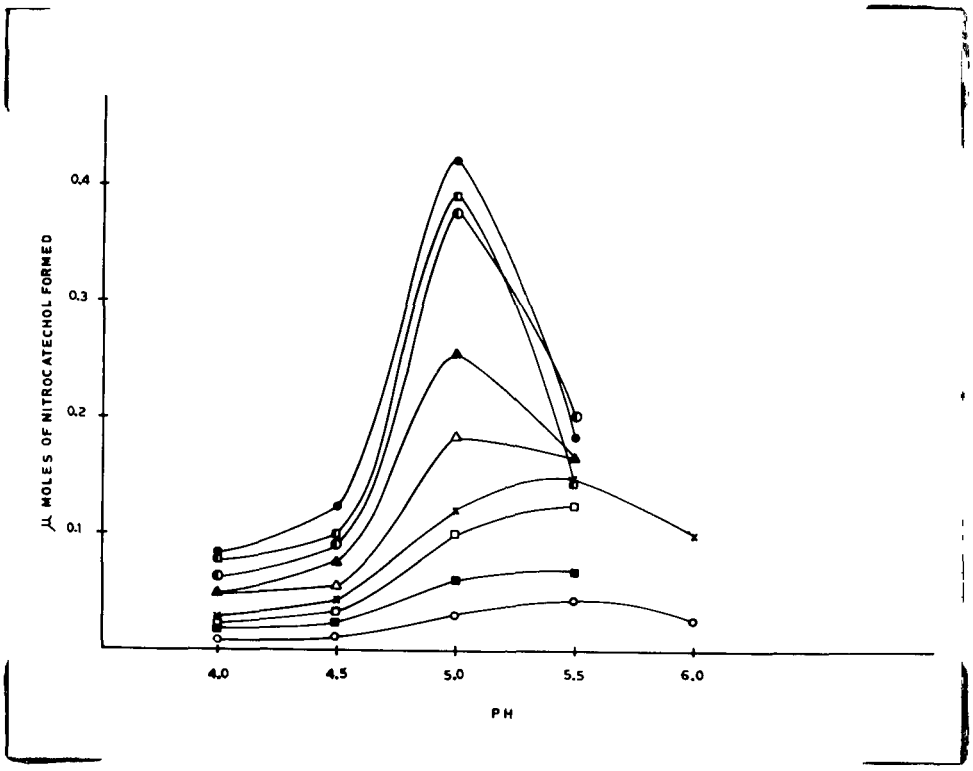

FIG.5. pH activity curve for chicken brain aryl-sulphatase A at various time of intervals. The assay mixture contained in a final volume of 0.1 ml, 0.2M-sodium acetate buffer of various pH values, 6mM p-nitrocatechol sulphate and 10 μg of enzyme protein. The reaction was terminated and nitrocatechol formed was determined as described in the Methods.

○, 5 min., ■, 10 min., □, 15 min.,

×, 20 min., △, 30 min., ▲, 45 min.,

◐, 60 min., ◧, 75 min., ●, 90 min.

after incubation for 5 min. (Fig.4).

Variation of enzyme concentration: Fig. 6 shows the effect of variation of enzyme protein on activity. There was a linear relationship between enzyme concentration and activity.

Effect of time of incubation on enzyme activity: The relation between enzyme activity and time of incubation at various pH values is shown in Fig.7. In no case was the activity proportional to the time of incubation over the whole incubation period. The pattern of the time-activity curve (Fig.8) of this enzyme was similar to that of other animal species as described in Chapter II.4.(iv). Harinath and Robins (35) did not observe any anomalous time-activity relationship for human brain arylsulphatase A using 4-methylumbelliferone sulphate as substrate. Chicken brain arylsulphatase A showed linear time-activity relationship in the presence of pyrophosphate as reported by Baum and Dodgson (32) for the human liver arylsulphatase A.

Effect of sulphite, phosphate and sulphate on enzyme activity: Sulphite, phosphate and sulphate ions showed marked inhibitory effect on enzyme activity. The nature of inhibition was competitive as shown in

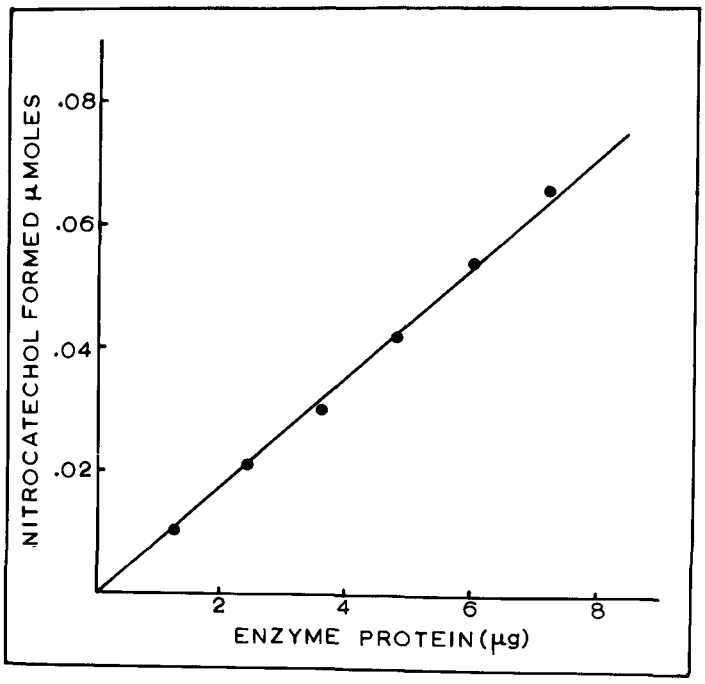

FIG.6. Nitrocatechol formation as a function of enzyme concentration. The assay mixture consisted of 0.2M-sodium acetate buffer pH 5.5, 6mM-p-nitrocatechol sulphate and various concentraions of enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as indicated in the Methods.

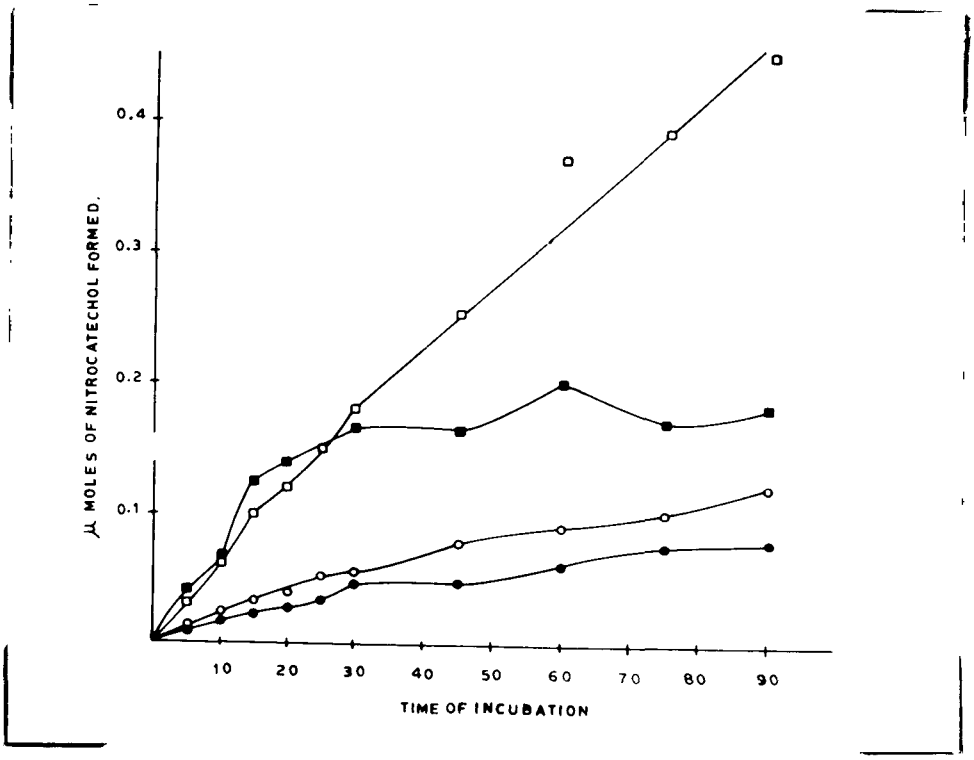

FIG.7. Nitrocatechol formation as a function of time at various pH values. The assay mixture consisted of 0.2M-sodium acetate buffer of various pH values, 6mM p-nitrocatechol sulphate and 10 µg of enzyme protein in a total volume of 0.1 ml. The reaction was terminated and nitrocatechol formed was determined as described in the Methods.

●, pH 4.0; ○, pH 4.5; □, pH 5.0; ■, pH 5.5.

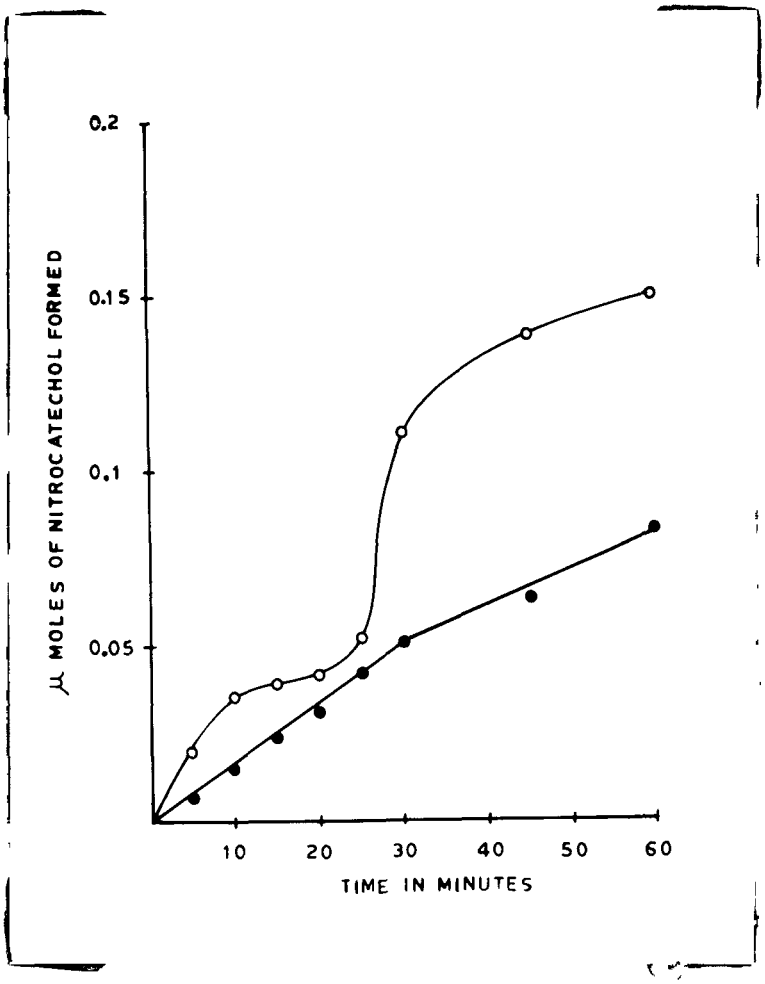

FIG.8. Time activity curve for chicken brain arylsulphatase A with and without pyrophosphate. The assay mixture consisted of 0.2M-sodium acetate buffer, pH 5.5, 6mM p-nitrocatechol sulphate and 2.4 μg of enzyme protein in a total volume of 0.1 ml. The reaction was terminated and nitrocatechol formed was determined as described in the Methods.

○, No addition; ●, 0.25mM sodium pyrophosphate.

Fig. 9,10,11. The Km value for p-nitrocatechol sulphate was 0.81 mM. The reported Km values for partially purified ox liver and human brain arylsulphatase A are 8 X 10^{-4}M and 1.54 X 10^{-4}M respectively (24,34). The Ki values for sulphite, phosphate and sulphate ions are shown in Table II. Harinath and Robins (35) found with 4-methylumbelliferone sulphate that the arylsulphatase A of human brain was competitively inhibited by sulphate ions.

Effect of pH on the stability of enzyme: The effect of pH on the stability of enzyme is shown in Table III. The enzyme is unstable on acidic pH i.e., 3.5 - 4.5 but quite stable above pH 5.0. Nichol and Roy (68,69,70) have reported that ox liver arylsulphatase A forms an insoluble tetramer at acidic pH (pH 5.0) and probably hydrophobic bonds are involved in the aggregation since it is reversed by dioxan as well as by raising the pH so that the electrostatic repulsion between similarly charged units increases.

Effect of cyanide, fluoride and chloride ions on enzyme activity: Potassium cyanide (10 mM) caused about 16% inhibition whereas sodium fluoride at this concentration caused 67% inhibition of enzyme activity (Table IV). Balasubramanian and Bachhawat (34) also

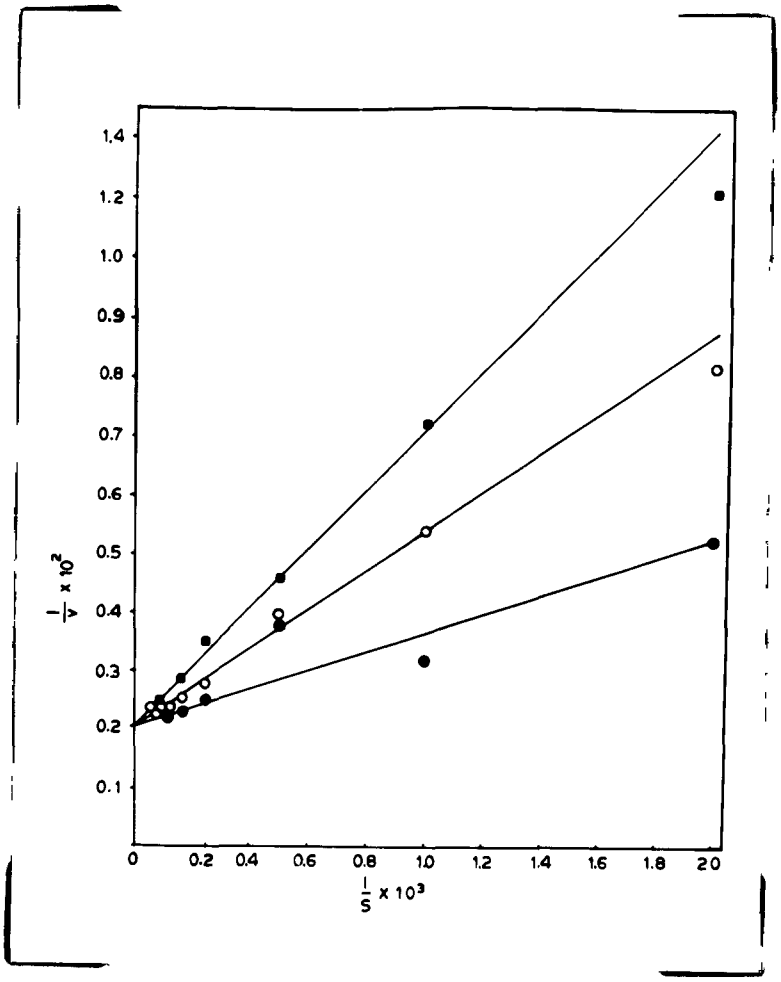

FIG.9. Lineweaver-Burk plots showing the effect of sodium sulphite on chicken brain arylsulphatase A. The assay mixture consisted of 0.2M-sodium acetate buffer pH 5.5, different concentrations of p-nitrocatechol sulphate and 4.8 μg of enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as indicated in the Methods.

●, No addition; ○, 1.25×10^{-5}M and ■, 2.5×10^{-5}M.

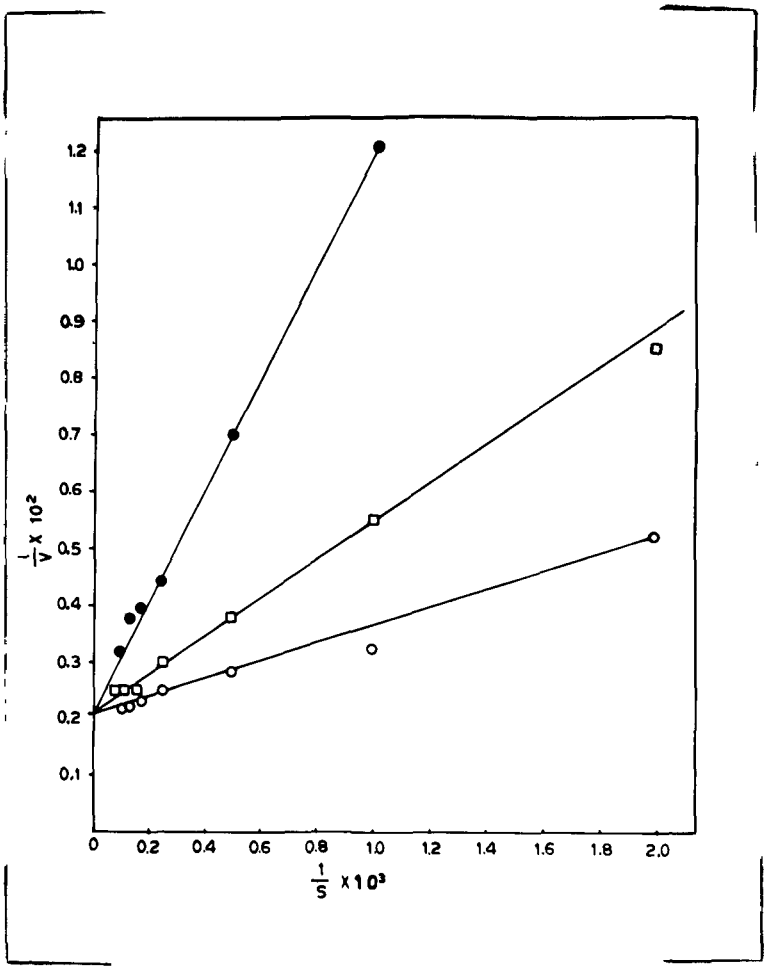

FIG.10. Lineweaver-Burk plots showing the effect of potassium phosphate on chicken brain arylsulphatase A. The assay mixture consisted of 0.2M-sodium acetate buffer pH 5.5, different concentrations of p-nitrocatechol sulphate and 4.8 µg of enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as indicated in the Methods.

○, No addition; □, 0.5×10^{-4}M and ●, 2.5×10^{-4}M.

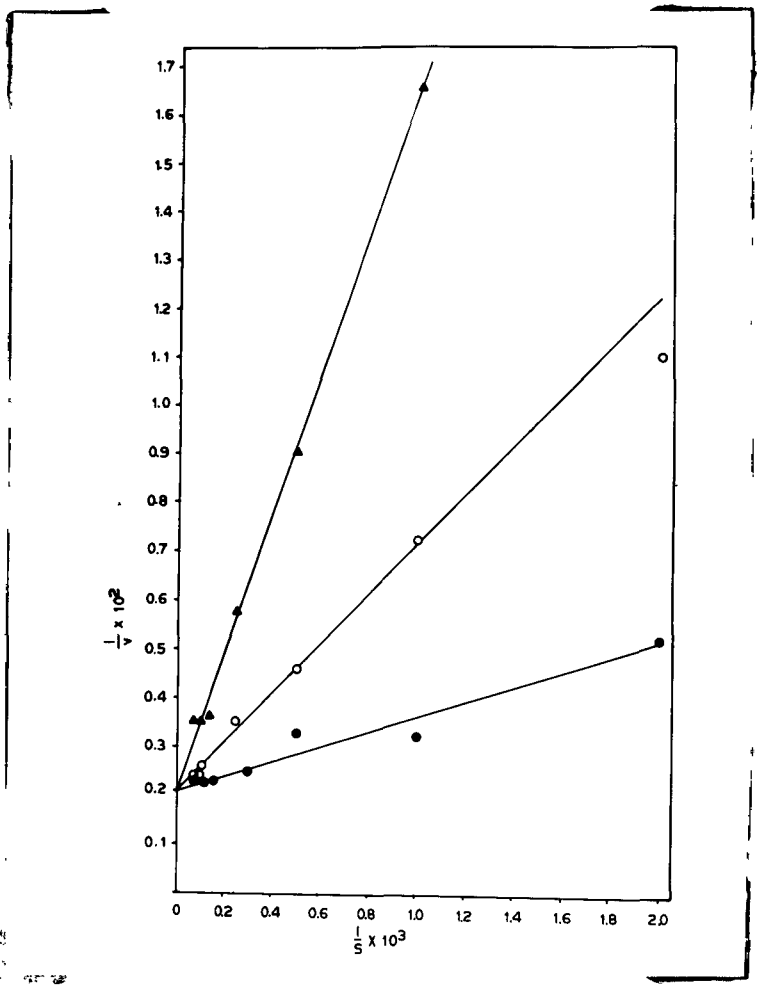

FIG.11. Lineweaver-Burk plots showing the effect of sodium sulphate on chicken brain arylsulphatase A. The assay mixture consisted of 0.2M-sodium acetate buffer pH 5.5, different concentrations of p-nitrocatechol sulphate and 4.8 µg of enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as indicated in the Methods.

●, No addition; ○, 2.5×10^{-2}M and △, 5×10^{-2}M.

CHAPTER III

TABLE II

THE Ki VALUES FOR SULPHITE, PHOSPHATE AND SULPHATE IONS

IONS USED	Ki VALUE
Sulphite	1.2×10^{-5} M
Phosphate	0.48×10^{-4} M
Sulphate	0.92×10^{-2} M

CHAPTER III

TABLE III

EFFECT OF pH VALUE ON THE STABILITY OF CHICKEN BRAIN ARYLSULPHATASE A

pH value	Nitrocatechol formed (nmol)
Control	96
3.5	15
4.0	40
4.5	40
5.0	70
5.5	80
6.0	85
6.5	85

The purified chicken brain arylsulphatase A was preincubated at 37°C for 1 h in 0.1M-Sodium acetate buffer of different pH values. The assay mixture consisted of 0.2 M-Sodium acetate buffer pH 5.5, 6mM-p-nitrocatechol sulphate and 9.6 µg enzyme protein in a total volume of 0.1ml. The reaction was terminated as described in the Methods. In control reaction mixture the enzyme was added without preincubation.

CHAPTER III

TABLE IV

EFFECT OF CYANIDE, FLUORIDE AND CHLORIDE IONS ON CHICKEN BRAIN ARYLSULPHATASE A

Component added	Concentration (mM)	Nitrocatechol formed (nmol)
Control	-	45
Cyanide	5	39
	10	38
Fluoride	5	18
	10	15
Chloride	5	42
	10	35

The purified chicken brain arylsulphatase A was kept at room temperature for 1 h in 0.2M-sodium acetate buffer pH 5.5 along with various concentration of anions as indicated above with occasional shaking. Then the reaction was started by the addition of nitrocatechol sulphate (6 mM) in a final volume of 0.1ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as indicated in the Methods. 4.8 µg enzyme protein was used. A control reaction mixture containing all the component except anions was treated in the same way.

found that fluoride ions caused marked inhibition compared with cyanide ions with human brain arylsulphatase A. Sodium chloride (10 mM) had no effect on enzyme activity.

Effect of pyrophosphate ions on enzyme activity: Table V shows the effect of pyrophosphate ions on enzyme activity. The enzyme is inhibited by pyrophosphate as reported by Baum and Dodgson (32).

Effect of citrate ions on enzyme activity: The effect of citrate ions on enzyme activity is shown in Table VI. The enzyme is inhibited by citrate ions at high concentration. Roy (24) reported that ox liver arylsulphatase B is inhibited by citrate ions.

Effect of carbonyl reagent, ascorbate and metal ions on enzyme activity: Like ox liver arylsulphatase A (123) both chicken and human brain arylsulphatase A were inhibited by carbonyl reagents (Table VII). The inhibition was more marked in the presence of 0.02 mM Cu^{2+} (Tables VIII, IX). Other metal ions such as Fe^{2+} and Zn^{2+} at 0.2 mM were not effective. In the absence of carbonyl reagents, Cu^{2+} inhibited the enzyme activity at a very high concentration (2 mM). Other metal ions such as Fe^{2+}, Zn^{2+} and Co^{2+} did not affect the enzyme activity at 0.02-2 mM (Table X). Bleszynski and

CHAPTER III

TABLE V

EFFECT OF SODIUM PYROPHOSPHATE ON CHICKEN BRAIN ARYLSULPHATASE A

Concentration (mM)	Nitrocatechol formed (nmol)
Control	48
0.100	42
0.125	35
0.250	29
0.500	22
10.000	14
20.000	6

The assay mixture consisted of 0.2-M sodium acetate buffer pH 5.5, 6mM-p-nitrocatechol sulphate, various concentrations of sodium pyrophosphate and 4.8 µg of enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min at 37°C and the reaction as terminated as described in the Methods. A control reaction mixture was run without sodium pyrophosphate.

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TABLE VI

EFFECT OF CITRATE IONS ON CHICKEN BRAIN ARYLSULPHATASE A

Sodium citrate concentration (mM)	Nitrocatechol formed (nmol)
Control	39
1	32
2	31
4	28
8	20

Conditions of assay were the same as described in Table V except different concentrations of sodium citrate were added.

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TABLE VII

EFFECT OF CARBONYL REAGENTS ON CHICKEN AND HUMAN BRAIN ARYLSULPHATASE A

Carbonyl reagents	Concentration	Nitrocatechol formed (nmol)	
		Chicken arylsulphatase A	Human arylsulphatase A
Control	-	45	45
Hydroxylamine	1 mM	28	20
	2 mM	20	15
Phenylhydrazine	1 mM	13	45
	2 mM	4	24
Hydrazine	0.5 mM	34	-
	1 mM	23	-

The purified chicken brain arylsulphatase A was treated with carbonyl reagents and its activity was assayed as described in Table IV. The purified human brain arylsulphatase A was treated similarly, except that it was kept with the carbonyl reagent at pH 5.0 for 30 min.; 55.6 µg enzyme protein was used, and the incubation was for 20 min. at 37°C . The control reaction mixtures contained all the components except carbonyl reagent, and p-nitrocatechol sulphate was added after 1 h (chicken) and 30 min. (human) under similar conditions.

TABLE VIII

EFFECT OF HYDROXYLAMINE AND METAL IONS ON CHICKEN AND HUMAN BRAIN ARYLSULPHATASE A

Component added	Concentration (mM)	Nitrocatechol formed (nmol)	
		Chicken aryl-sulphatase A	Human aryl-sulphatase A
Control	-	40	45
Hydroxylamine	1	30	20
Hydroxylamine + Cu^{2+}	0.02	6	14
Hydroxylamine + Fe^{2+}	0.20	27	29
Hydroxylamine + Zn^{2+}	0.20	27	-
Hydroxylamine + Co^{2+}	0.20	30	-

The enzyme was kept at room temperature in sodium acetate buffer, pH 5.5 (chicken) and pH 5.0 (human) with metal ion as indicated and (1 mM) Hydroxylamine, it was assayed as described in Table VII, 4.8 µg (chicken) and 55.6 µg (human) enzyme protein was used. In the control reaction mixture none of the component was added.

TABLE IX

EFFECT OF PHENYLHYDRAZINE AND METAL IONS ON CHICKEN AND HUMAN BRAIN ARYLSULPHATASE A

Component added	Concentration (mM)	Nitrocatechol formed (nmol)	
		Chicken aryl-sulphatase A	Human aryl-sulphatase A
Control	-	40	45
Phenylhydrazine	0.5	28	45
Phenylhydrazine + Cu^{2+}	0.02	20	6
Phenylhydrazine + Cu^{2+}	0.2	18	0
Phenylhydrazine + Fe^{2+}	0.2	45	30
Phenylhydrazine + Zn^{2+}	0.2	22	-
Phenylhydrazine + Co^{2+}	0.2	24	-

The enzyme was assayed as indicated in Table VIII except 0.5mM phenylhydrazine in place of hydroxylamine was used.

TABLE X

EFFECT OF EDTA AND METAL ION ON CHICKEN AND HUMAN ARYLSULPHATASE A

Component added	Chicken Arylsulphatase A		Human arylsulphatase A	
	Concentration (mM)	Nitrocatechol formed (nmol)	Concentration (mM)	Nitrocatechol formed (nmol)
Control	-	45	-	45
EDTA	2.50	43	2.50	42
	5.00	42	5.00	41
Cu^{2+}	0.05	46	0.02	47
	0.50	22	1.00	45
	2.00	5	2.00	33
Fe^{2+}	0.50	38	1.00	46
	2.00	42	2.00	43
Co^{2+}	0.50	42	-	-
	2.00	39	-	-
Ag^{+}	0.025	54	-	-
	0.250	52	-	-
	0.500	45	-	-
	1.000	45	-	-

The chicken and human brain arylsulphatase A were treated with EDTA and metal ions and their activity was assayed as described in Tables IV and VII.

Leznicki (73) and Harinath and Robins (35) reported that silver nitrate (0.25mM) produced 96% inhibition of arylsulphatase A whereas it stimulated the activity of arylsulphatase B. With chicken brain arylsulphatase A silver nitrate (0.025-1mM) did not inhibit enzyme activity and a low concentration (0.025mM) stimulated the enzyme activity by 20% (Table X). Ascorbate did not inhibit the activity of either chicken or human brain arylsulphatase A. The inhibition by Cu^{2+} was enhanced by ascorbate. Other metal ions such as Fe^{2+}, Zn^{2+} (0.02-0.2mM) did not affect the enzyme activity even in the presence of ascorbate (Table XI).

Effect of cysteine and p-hydroxymercuribenzoate on enzyme activity: Cysteine did not have any appreciable effect on the enzyme activity. p-Hydroxymercuribenzoate (1mM) and N-ethylmaleimide (2mM) did not affect the enzyme activity at pH 5.5, which was the optimum pH for the enzyme activity but at pH 5.0 considerable inhibition was observed (Table XII). Like chicken brain arylsulphatase A, human brain arylsulphatase A is also inhibited by p-hydroxymercuribenzoate at pH 5.0 (Table XIII). The inhibition by p-hydroxymercuribenzoate at pH 5.0 was reversed by cysteine in both the cases. These findings agree with those of

TABLE XI

EFFECT OF ASCORBATE AND METAL IONS ON CHICKEN AND HUMAN ARYLSULPHATASE A

Ascorbate metal ion	Concentration (mM)	Nitrocatechol formed (nmol)	
		Chicken arylsulphatase A	Human arylsulphatase A
Control	-	40	45
Ascorbate	1	40	48
Ascorbate + Cu^{2+}	0.02	5	36
Ascorbate + Cu^{2+}	0.2	0	18
Ascorbate + Fe^{2+}	0.2	43	-
Ascorbate + Zn^{2+}	0.2	46	-
Ascorbate + Co^{2+}	0.2	35	-

The enzyme was assayed as described in Tables VIII and IX except 1mM Ascorbate was used in place of carbonyl reagents.

CHAPTER III

TABLE XII

EFFECT OF CYSTEINE, p-HYDROXYMERCURIBENZOATE AND N-ETHYLMALEIMIDE ON CHICKEN BRAIN ARYLSULPHATASE A

Component added	Concentration (mM)	Nitrocatechol formed (nmol)	
		pH 5.0	pH 5.5
Control	-	24	38
Cysteine	1.0	-	40
	2.5	-	33
p-Hydroxymercuribenzoate	0.5	16	41
	1.0	8.4	33
p-Hydroxymercuribenzoate + Cysteine	0.5 + 10.0	24	-
p-Hydroxymercuribenzoate + Cysteine	1.0 + 10.0	24	-
N-Ethylamaleimide	1.0	24	38
	2.0	13	38

The purified chicken brain arylsulphatase A was kept at room temperature for 1 h in 0.2 M-sodium acetate buffer pH 5.0 and 5.5 along with required concentration of component with occasional shaking. In studies on the reversal of p-hydroxymercuribenzoate inhibition by cysteine, the neutralised cysteine was added just before starting the reaction by the addition of nitrocatechol sulphate (6 mM) in a final volume of 0.1 ml. In control reaction mixture none of the component was added. Other conditions were the same as described in Table IV.

CHAPTER III

TABLE XIII

EFFECT OF p-HYDROXYMERCURIBENZOATE AND CYSTEINE ON HUMAN ARYLSULPHATASE A

p-Hydroxymercuribenzoate (m')	Nitrocatechol formed (nmol)
Control	48.0
0.1	54.0
0.5	13.2
1.0	7.2
0.5 p-Hydroxymercuri-zoate + 5 cysteine	40.8
0.5 p-Hydroxymercuri-zoate + 10 cysteine	45.0

The purified human brain arylsulphatase A was kept at room temperature for 30 min. in 0.2 M-sodium acetate buffer pH 5.0 along with required concentration of p-hydroxymercurizoate with occasional shaking. The other conditions were the same as described in Table XII.

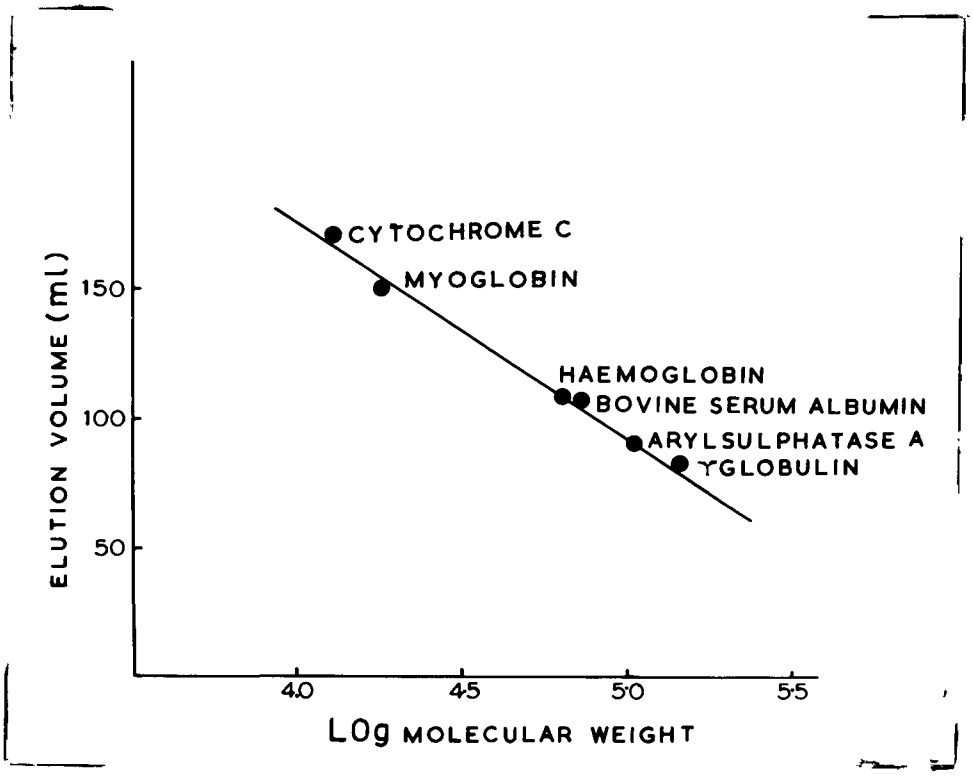

FIG.12. The determination of molecular weight of chicken brain arylsulphatase A by gel filtration on Sephadex G-200. The size of the column and other conditions are described in the Methods.

Jerfy and Roy (53) who reported that ox liver enzyme at high pH values was not inhibited by p-chloromercuribenzoate but at lower pH values it was, and this inhibition could be reversed by cysteine. Balasubramanian and Bachhawat (34) found that p-chloromercuribenzoate could inhibit arylsulphatase A of human brain but this inhibition could not be reversed by cysteine at pH 4.5. Bleszynski and Leznicki (73) found that ox brain arylsulphatase A was markedly inhibited by p-chloromercuribenzoate but this inhibition could not be reversed by glutathione. Harinath and Robins (35) observed 77% inhibition of human brain arylsulphatase A by 0.1 mM p-chloromercuribenzoate. These authors did not see if thiol reagents restored enzyme activity.

Molecular weight: The molecular weight of purified chicken brain arylsulphatase A (Fig.12) was found to be 104,700 which is almost similar to arylsulphatase A of ox liver (69), human brain (35) and human placenta (74).

5. Discussion:

The behaviour of the purified chicken brain arylsulphatase, especially the Km value, anomalous time-activity relationship, inhibitory effect of sulphite,

phosphate and sulphate ions and molecular weight suggested that it might be classified as an arylsulphatase A. Like human liver arylsulphatase A (20) two pH optima were obtained at different time of incubation, i.e. pH 5.5 for 5-20 min. and pH 5.0 for 30-90 min. Balasubramanian and Bachhawat (34) found only one pH optimum, 4.5, for 10 min. incubation for human brain arylsulphatase A. Harinath and Robins (35) also reported only one pH optimum, 5.7, at different times of incubation for human brain arylsulphatase A with 4-methylumbelliferone sulphate as substrate. Further, the chicken brain arylsulphatase showed an anomalous time-activity relationship and the shape of time-activity curve markedly changed in the presence of pyrophosphate ions as reported by Baum and Dodgson (32). Harinath and Robins (35) using 4-methylumbelliferone sulphate as substrate found that the human brain arylsulphatase A did not show any anomalous time-activity relationship. These differences in the kinetic properties may be attributed to the different type of substrate employed for the kinetic studies. Except for Cu^{2+}, other metal ions employed in the present study had no inhibitory effect on the enzyme activity. Moreover, Ag^{+}, which was reported to be inhibitory for ox and human brain arylsulphatase A (35,73) was found to have a stimulatory effect on

chicken brain arylsulphatase A.

The inhibition of chicken and human brain arylsulphatase A by carbonyl reagents in the presence of traces of Cu^{2+} was in agreement with Roy's (123) work. The actual mechanism of inhibition is not known. However, Roy (123) had suggested two possible mechanisms for this inhibition. In the first mechanism he proposed that the enzyme reacted with Cu^{2+} and the carbonyl reagent to give a ternary complex, which was enzymatically inactive. In the second possibility Cu^{2+} was reduced to Cu^{+} by the carbonyl reagent and the latter was the actual inhibitor of enzyme activity. Ascorbate alone had no effect on enzyme activity in our system, but it enhances the inhibition by Cu^{2+}. The second mechanism of inhibition as postulated by Roy (123)therefore seems to be more probable.

Chicken brain arylsulphatase A, however, resembled arylsulphatase B of other animal species in its electrophoretic mobility, behaviour under zinc acetate fractionation,the stimulation of enzyme activity by silver nitrate and inhibition by citrate ions. Roy (50) had reported that livers of marsupials and lower mammals which were more closely related to birds during evolution, contain a single type II arylsulphatase

with properties intermediate between those of arylsulphatases A and B of ox liver. Red-kangaroo liver arylsulphatase A, which was purified by Roy (71) showed less pronounced anomalous kinetics, did not polymerize at low pH values, had a different isoelectric point and different Ki for sulphate ion compared with arylsulphatase A of ox liver. Thus, the chicken brain arylsulphatase did not correspond to either arylsulphatase A or arylsulphatase B but rather had combined properties of both.

6. Summary:

Chicken brain arylsulphatase A was purified 2000-fold with overall recovery of 14%, by using ammonium sulphate fractionation, ethanol precipitation, Sephadex G-200 gel filtration and DEAE-Sephadex column chromatography. The purified preparation was free from β-glucuronidase, β-galactosidase, acid phosphatase, inorganic pyrophosphatase and adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase activities. The enzyme has molecular weight of 104,700. Polyacrylamide gel-electrophoresis indicated that the purified preparation was not homogeneous. Chicken brain arylsulphatase was markedly inhibited by carbonyl reagents in the presence of traces of Cu^{2+} in the system. Other

metal ions such as Fe^{2+} and Zn^{2+}, were inactive. Ascorbic acid alone had no effect on enzyme activity but enhances the inhibition by Cu^{2+}. Chicken brain arylsulphatase A resembled arylsulphatase A of other animal species in its kinetic properties such as Km value, anomalous time-activity relationship and inhibitory effect of phosphate, sulphite and sulphate ions. However, its electrophoretic mobility, behaviour under zinc acetate fractionation, stimulation by Ag^{+} and inhibition by citrate ions were similar to arylsulphatase B of other animal species. Thus, this enzyme did not correspond to either arylsulphatase A or arylsulphatase B but properties of both.

CHAPTER IV.

ENZYMATIC DESULPHATION OF CEREBROSIDE 3-SULPHATE BY CHICKEN BRAIN ARYLSULPHATASE A.

CHAPTER IV.

ENZYMATIC DESULPHATION OF CEREBROSIDE 3-SULPHATE BY CHICKEN BRAIN ARYLSULPHATASE A.

Chapter II has indicated the differences in proportions and physicochemical properties of brain arylsulphatases in various animal species. Chicken brain arylsulphatase A in some respects resembles arylsulphatase A of other animal species in its kinetic properties such as Km value, anomalous time-activity relationship and inhibitory effect of sulphite, phosphate and sulphate ions. On the other hand its electrophoretic mobility and behaviour during zinc acetate fractionation are similar to those of arylsulphatase B of other species.

Mehl and Jatzkewitz (79) have shown that arylsulphatase A from pig kidney can degrade cerebroside 3-sulphate in the presence of a heat stable factor. However, at present there is no information available as to whether or not the brain arylsulphatase A can degrade cerebroside 3-sulphate. The present chapter describes the properties of chicken brain arylsulphatase A using cerebroside 3-sulphate and p-nitrocatechol sulphate as substrate.

1. Materials:

Dipotassium salt of p-nitrocatechol sulphate was purchased from Sigma Chemical Co., (St.Louis, Mo., USA), Aquacide I (Polyethelene glycol) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Triton X-100 from Rohm and Hass (USA), sodium deoxycholate from British Drug Houses Ltd., (England), Brij 96 from Atlas Chemical Co., (USA), Tween 20 and 80 from Mann Research Laboratories (USA) and Cutscum from Fisher Scientific Company, U.S.A. Florosil 60-100 mesh from Floridin Co., U.S.A. Silica gel G from E. Merk, Germany. Cerebroside 3-sulphate from Biochemical Unit, V.P. Chest Institute, New Delhi. (^{35}S) Sodium sulphate was obtained from the Atomic Energy Establishment, Trombay, India and all other chemicals used were of analytical grade. All organic solvents were distilled before use.

2. Methods:

2.(i) Arylsulphatase A assay using (^{35}S) cerebroside 3-sulphate as substrate: The reaction mixture contained 0.01M-sodium acetate buffer pH 4.5, 70,000 c.p.m. cerebroside 3-sulphate (22 nmol) and 30 µg enzyme protein (0.76 unit) in a total volume of 0.1 ml. The tubes

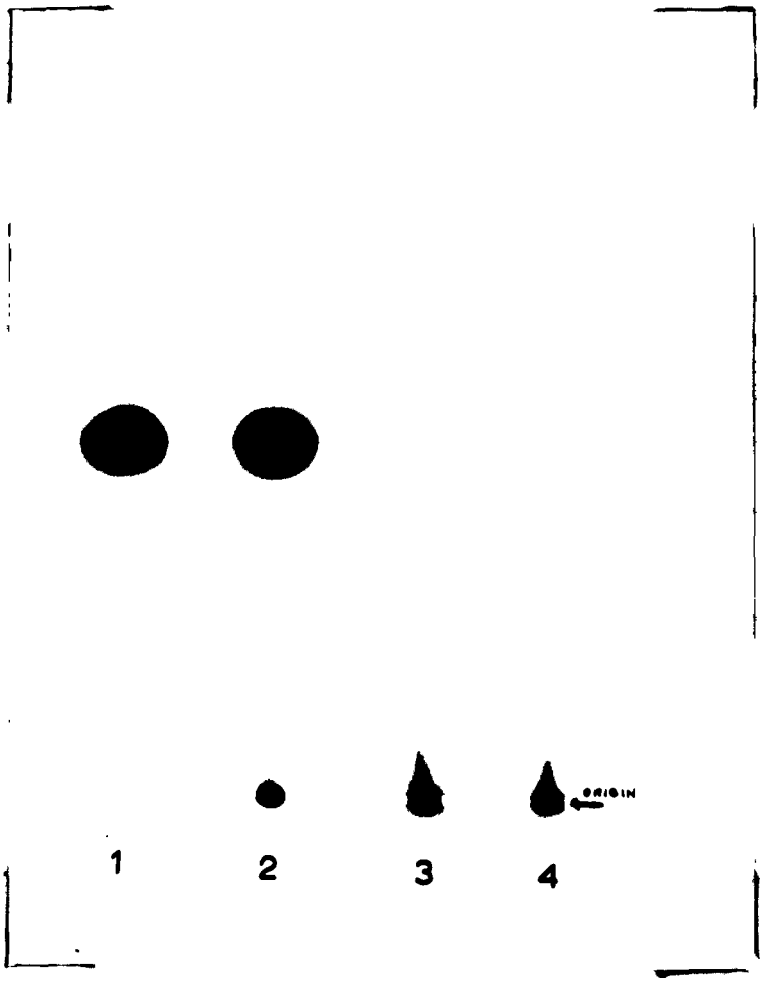

FIG.1. Radioautogram showing the electrophoretic separation of (^{35}S) sulphate from (^{35}S) cerebroside 3-sulphate. The conditions of assay were the same as described in the Methods.

1. Standard (^{35}S) sulphate;
2. Reaction mixture;
3. Control reaction mixture;
4. (^{35}S) Cerebroside 3-sulphate.

were incubated for 1 h at 37°C and the reaction was terminated by heating the tubes in a boiling water bath for 30 sec. Immediately after heating, the tubes were cooled in ice. The precipitated protein was removed by centrifugation. The separation of (^{35}S) sulphate and undegraded (^{35}S) cerebroside 3-sulphate was achieved by the paper electrophoresis using Whatman 3MM paper (18 X 35 cm). An aliquot of the supernatant was streaked on the paper in the form of 1 cm. band. The paper electrophoresis was carried out in 0.03M-sodium barbitone buffer pH 8.0 for 1.5 h at 250 volts. Under these conditions the distance moved by the standard (^{35}S) sulphate as well as liberated sulphate was the same (11 cm) and undegraded cerebroside 3-sulphate stayed at the origin (Fig. 1).

The areas corresponding to (^{35}S) sulphate were cut out from the paper and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter as described in Chapter III.3.(iii). In the control reaction mixture the enzyme was added at the end of the incubation period.

2.(ii) Arylsulphatase A assay using p-nitrocatechol sulphate as substrate: The arylsulphatase A assay was done as described in Chapter III.3.(i) using 5 min. incubation period.

2.(iii) Isolation of cerebroside 3-(^{35}S) sulphate: The rats (17 days old) were injected intraperitoneally with carrier-free $Na_2{}^{35}SO_4$ (20 μCi/g body wt.) and killed after 24 h. The brains were removed and homogenised in chloroform-methanol (2:1 v/v) (19 ml/g of wet tissue) in a Potter-Elehjem homogeniser for 5 min. and the homogenate was filtered. The filtrate was partitioned with 0.2 vol. of 0.74M-KCl and then the lower phase was washed twice with equal volumes of theoretical upper phase by the method of Folch et al (124). The lower phase was concentrated to dryness under vacuum and passed through a Florisil column by the method of Rouser et al (125). The cerebroside and cerebroside 3-sulphate were eluted with chloroform-methanol (7:3, v/v) and concentrated under vacuum at 40°C. The cerebroside and cerebroside 3-sulphate were separated by DEAE-cellulose acetate chromatography by the method of Rouser et al (126).

2.(iv) Estimation and identification of cerebroside 3-sulphate: The cerebroside 3-sulphate was estimated by the method of Roughan and Batt (127) using phenol-sulphuric acid. The purity of (^{35}S) cerebroside 3-sulphate was checked by thin layer chromatography. Thin layer chromatography plates (20 cm X 20 cm) of

0.25 mm thickness were prepared using silica gel G. The plates were activated at 100°C for 1 h. The sample was dissolved in chloroform-methanol (2:1) and spotted on the plate. Ascending chromatography was done using the solvent system chloroform-methanol-water (14:6:1) (128) in a closed chamber presaturated with solvent vapours. After the chromatography, the plates were dried at room temperature, sprayed with 50% sulphuric acid and heated at 150°C for 20 min. Black spots appeared on the white background. The mobility of prepared cerebroside 3-sulphate was the same as that of standard cerebroside 3-sulphate. The specific radioactivity of the isolated cerebroside 3-sulphate was 3.5×10^6 c.p.m./mg. The solution of cerebroside 3-sulphate was prepared in 0.2% Tween 20 (v/v) and used as substrate.

2.(v) Preparation of enzyme: Chicken brain arylsulphatase A was purified as described in Chapter III.3(xi).

2.(vi) Concentration of enzyme by Aquacide I: The fraction eluted by 0.1 M-NaCl from the DEAE-Sephadex column was concentrated by placing it in a dialysis bag which was covered by Aquacide I and kept at 4°-6°C for 8 h. By this procedure ten-fold concentration of the enzyme activity was achieved. The concentrated enzyme was dialysed

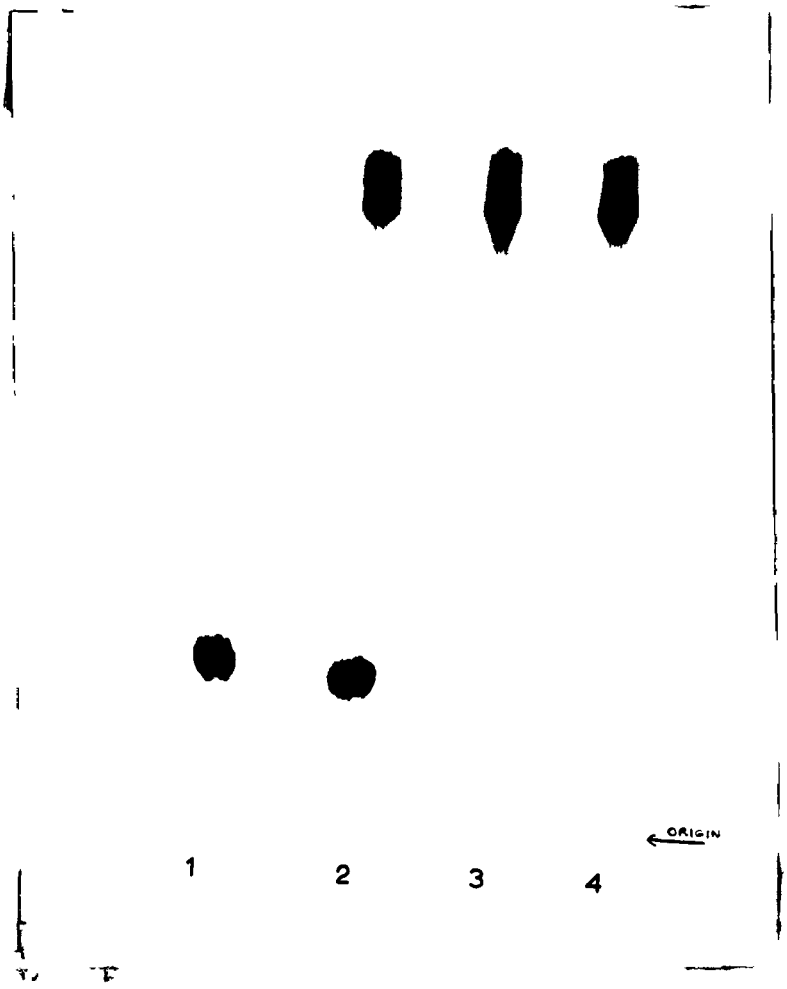

FIG.2. Radioautogram showing the paper chromatographic separation of (^{35}S) sulphate from (^{35}S) cerebroside 3-sulphate. Conditions of assay were the same as described in the Methods.

1. Standard (^{35}S) sulphate;
2. Reaction mixture;
3. Control reaction mixture;
4. (^{35}S) Cerebroside 3-sulphate.

against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4, for 6 h and was used to study the degradation of cerebroside 3-sulphate.

2.(vii) Paper chromatography: An aliquot of the supernatant was streaked in form of a band 1cm on Whatman 3MM paper. Descending chromatography was done in isobutyric acid 0.5M-ammonia (5/3, v/v) (129). Area corresponding to (^{35}S) sulphate was cut out from the paper and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter as described in Chapter III.3(iii). The reaction product and standard sulphate had Rf value 0.28 (Fig.2) while undegraded cerebroside 3-sulphate moved to the solvent front.

3. Results:

As mentioned in Chapter III.4. [redacted] the enzyme preparation was not homogeneous. It had two protein bands and major band had arylsulphatase A activity. To check whether cerebroside 3-sulphatase activity resided in the same band or not, the major band was cut out from several gels, the eluted protein was concentrated by Aquacide I treatment and dialysed. This eluted enzyme also had cerebroside 3-sulphate degrading activity (Table I). The enzyme activity recovered from

CHAPTER IV

TABLE I

THE RECOVERY OF ENZYME ACTIVITY TOWARDS CEREBROSIDE 3-SULPHATE AND p-NITROCATECHOL SULPHATE BEFORE AND AFTER ELECTROPHORESIS

Substrate used	Before electrophoresis	After electrophoresis	Recovery from the Gel
p-Nitrocatechol sulphate	15 units*	5 units	33
Cerebroside ** 3-sulphate	61560 c.p.m.	19500 c.p.m.	31

*Enzyme unit is defined in Chapter III (Table I).

**For cerebroside 3-sulphate the assay mixture consisted of 0.2M-sodium acetate buffer pH 5.0, 70,000 c.p.m. (^{35}S)cerebroside 3-sulphate (22 nmol) in water and 0.65 unit of enzyme in a total volume of 0.2 ml. The reaction was stopped as indicated in the Methods and an aliquot (0.1 ml) was streaked on Whatman 3MM paper and chromatography was done by the method of Suzuki and Strominger (Ref. No.129).

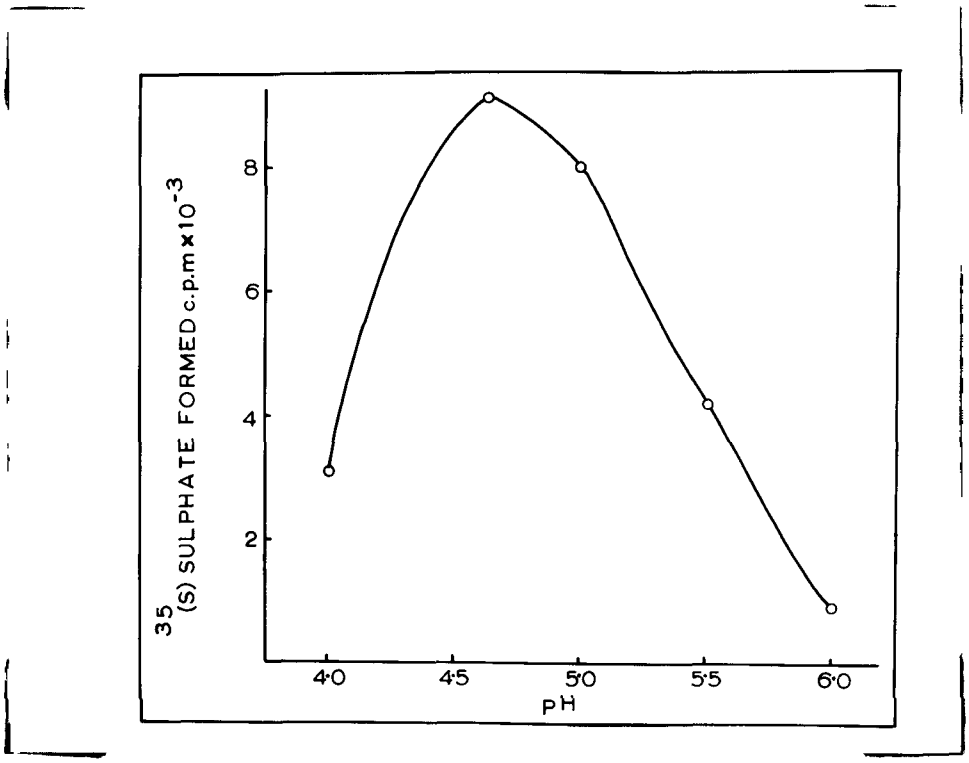

FIG.3. pH optimum of chicken brain aryl-sulphatase A using cerebroside 3-sulphate as substrate. Assay conditions were the same as described in the Methods except that sodium acetate buffer of various pH values was used.

the gel was 30% with cerebroside 3-sulphate as well as p-nitrocatechol sulphate.

3.(i) Identification of the product: The product of the reaction was identified as (^{35}S) sulphate by paper electrophoresis (Fig. 1) and paper chromatography (Fig. 2).

3.(ii) Properties of the enzyme:

Effect of detergent on enzyme activity:

Non-ionic detergents like Tween 20, Tween 80 and Brij 96, activate the enzyme activity approximately 3-fold when cerebroside 3-sulphate was used as substrate (Table II). These detergents also activate the activity of enzyme towards p-nitrocatechol sulphate. Sodium taurodeoxycholate has no effect on cerebroside 3-sulphate degradation but it slightly activates the p-nitrocatechol sulphate degradation. Deoxycholate inhibits the enzyme activity towards cerebroside 3-sulphate as well as p-nitrocatechol sulphate.

pH optimum: The enzyme showed maximum activity towards cerebroside 3-sulphate at pH 4.5 in acetate buffer (Fig. 3). The pH optimum for p-nitrocatechol sulphate degradation in acetate buffer was 5.5 as described in Chapter III.4.(i).

CHAPTER IV

TABLE II

EFFECT OF DETERGENTS ON CHICKEN BRAIN ARYLSULPHATASE A

Detergent	Sulphate released from cerebroside 3-sulphate (nmol)	Nitrocatechol formed from nitrocatechol sulphate (nmol)
None	0.94	36
Tween 20	2.50	49
Tween 80	2.70	48
Brij 96	2.54	45
Sodium Taurodeoxycholate	0.92	42
Sodium deoxycholate	0.40	24

For cerebroside 3-sulphate the assay mixture consisted of 0.01 M-sodium acetate buffer pH 4.5, 70,000 c.p.m. (^{35}S) cerebroside 3-sulphate (22 nmol), 0.2% detergents and 30 µg enzyme protein (0.76 unit) in a total volume of 0.1 ml. The tubes were incubated for 1 hr. at 37°C and the reaction was terminated as indicated in the Methods. For p-nitrocatechol sulphate the assay mixture consisted of 0.2 M-sodium acetate buffer pH 5.5, 6 mM p-nitrocatechol sulphate, 0.2% detergents and 3.37 µg enzyme protein in a total volume of 0.1 ml. The tubes were incubated for 5 min. at 37°C and the reaction was terminated as described in Chapter III.3.(i).

Effect of time of incubation: With cerebroside 3-sulphate as substrate the enzyme shows a linear increase in (^{35}S) sulphate formation upto 60 min. and then the curve plateau's off (Fig.4). This linear time-activity relationship can be compared with that obtained by Harinath and Robins (35) using 4-methylumbelliferone sulphate as substrate.

Variation of enzyme concentration: Fig.5 shows the effect of variation of enzyme protein on activity. There was a linear relationship between enzyme concentration and activity.

Variation of substrate concentration: The variation of (^{35}S) sulphate formation with increasing amounts of cerebroside 3-sulphate is shown in Fig.6. There was a linear increase in sulphate formation upto 0.33mM concentration of cerebroside 3-sulphate. However, at 0.55mM there was decrease in enzyme activity. The Km values and the Vmax for both cerebroside 3-sulphate and p-nitrocatechol sulphate degradation are shown in Table III.

Effect of sulphite, phosphate and sulphate ions on enzyme activity: The cerebroside 3-sulphate

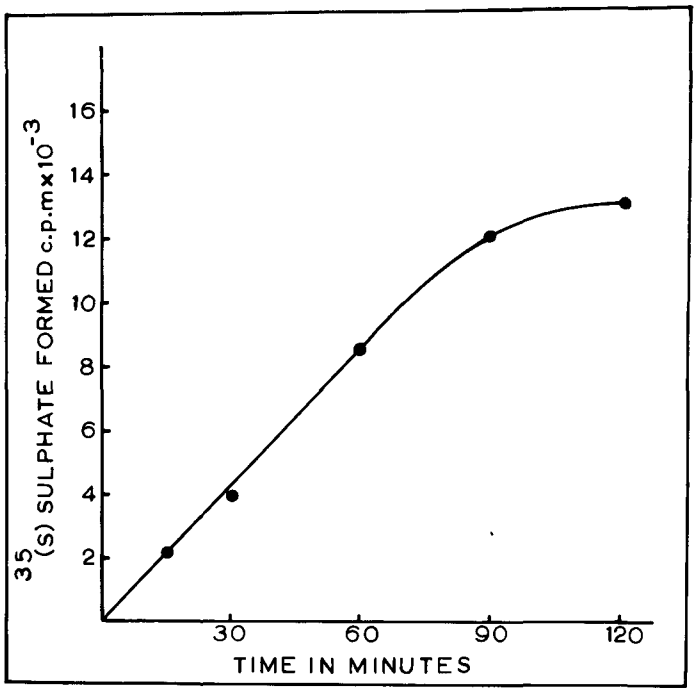

FIG. 4. The time-course of (^{35}S) sulphate formation. Assay conditions were the same as described in the Methods except that incubation period was varied as shown in the figure.

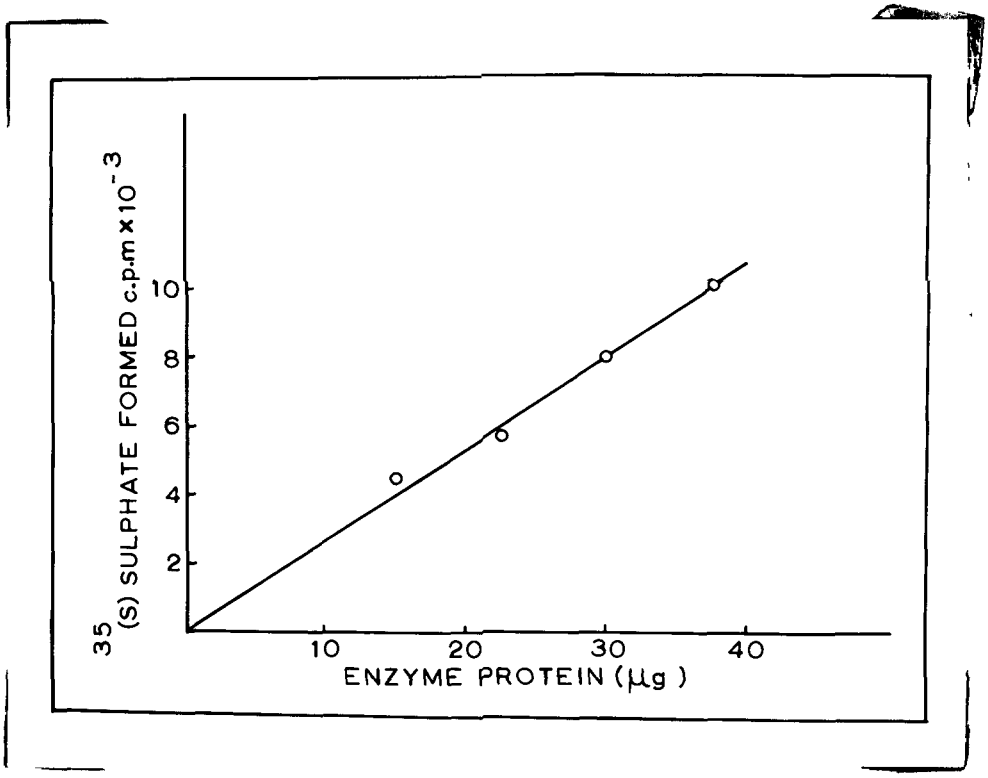

FIG. 5. (^{35}S) Sulphate formation as a function of enzyme concentration. Assay conditions were the same as described in the Methods except that the enzyme concentration was varied as shown in the figure.

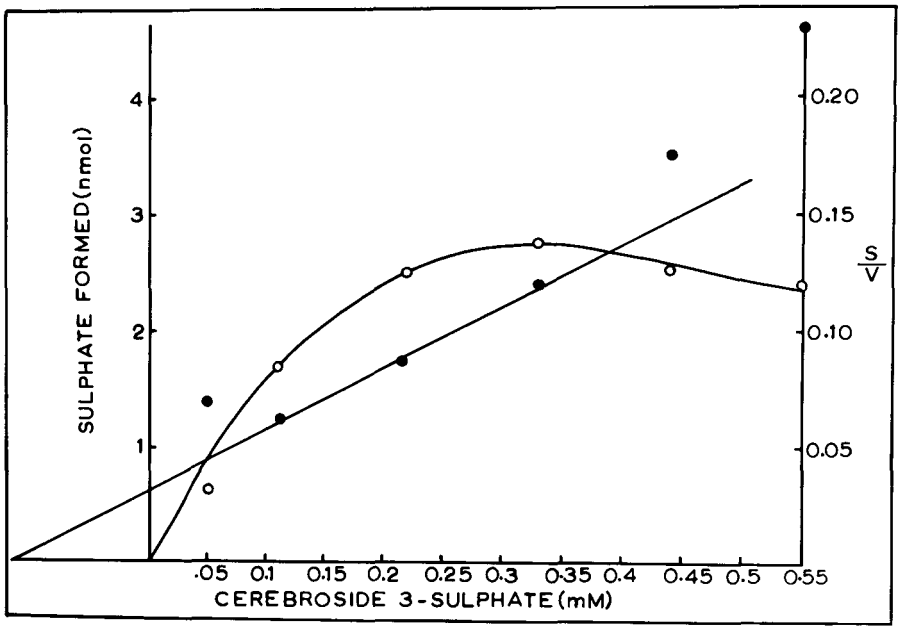

FIG.6. Effect of (^{35}S) cerebroside 3-sulphate on (^{35}S) sulphate formation. Assay conditions were the same as described in the Methods except that (^{35}S) cerebroside 3-sulphate concentration was varied as indicated in the figure.

CHAPTER IV

TABLE III

SOME KINETIC PARAMETERS FOR THE CHICKEN BRAIN ARYLSULPHATASE A

Parameter	Cerebroside 3-sulphate	p-Nitrocatechol sulphate
pH	4.50	5.50
Km (mM)	0.12	0.81
Vmax (nmol/min/mg)	1.50	2500

degrading activity of chicken brain arylsulphatase A is inhibited by sulphite, phosphate and sulphate ions (Tables IV, V, VI). The sulphite ions were powerful inhibitor compared to phosphate and sulphate ions. In the case of p-nitrocatechol sulphate degradation these ions competitively inhibited the enzyme activity as described in Chapter III.1. It should be noted here that arylsulphatase A activity towards p-nitrocatechol sulphate and cerebroside 3-sulphate is stimulated by low concentration of sulphate ions, and the activity towards p-nitrocatechol sulphate requires higher concentration of sulphate for inhibition compared to that of cerebroside 3-sulphate. Mehl and Jatzkewitz (80) found that pig kidney cerebroside sulphatase is inhibited by sulphite, phosphate and sulphate ions but not by cyanide ions.

4. Discussion:

In the present study it was observed that the purified chicken brain arylsulphatase A had cerebroside 3-sulphate degrading activity in the absence of a heat-stable polymer. This degradation of cerebroside 3-sulphate in the absence of a heat-stable polymer is particularly notable because this enzyme had certain different properties compared with arylsulphatase A of other animal

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TABLE IV

EFFECT OF SODIUM SULPHITE ON CEREBROSIDE 3-SULPHATE AND p-NITROCATECHOL SULPHATE DEGRADATION

Sulphite (mM)	Sulphate released from cerebroside 3-sulphate (nmol)	Nitrocatechol formed from nitrocatechol sulphate (nmol)
None	2.80	48.0
0.01	2.44	39.0
0.05	1.34	17.4
0.10	0.60	12.0
0.20	0.29	-
0.50	0.08	-

The assay mixture consisted of 0.01M-sodium acetate buffer pH 4.5, 70,000 c.p.m. (^{35}S)cerebroside 3-sulphate (22 nmol) in 0.2% Tween 20, indicated concentrations of sodium sulphite and 30 µg enzyme protein (0.76 unit) in a total volume of 0.1 ml. The reaction was terminated as indicated in the Methods.

The enzyme activity towards p-nitrocatechol sulphate was estimated for 5 min. incubation using 4.8 µg enzyme protein as described in Chapter III.3.(i). A control reaction mixture was run without sodium sulphite in both the cases.

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TABLE V

EFFECT OF POTASSIUM PHOSPHATE ON CEREBROSIDE 3-SULPHATE AND p-NITROCATECHOL SULPHATE DEGRADATION

Phosphate (ml)	Sulphate released from cerebroside 3-sulphate (nmol)	Nitrocatechol formed from nitrocatechol sulphate (nmol)
None	2.77	48.0
0.05	2.49	-
0.20	1.54	-
0.50	0.65	31.8
1.00	0.50	19.2
2.00	0.46	-

The assay mixture consisted of 0.01M-sodium acetate buffer pH 4.5, 70,000 c.p.m. (^{35}S) cerebroside 3-sulphate (22 nmol) in 0.2% Tween 20, indicated concentrations of potassium phosphate and 30 µg enzyme protein (0.76 unit) in a total volume of 0.1 ml. The reaction was terminated as indicated in the Methods.

The enzyme activity towards p-nitrocatechol sulphate was estimated for 5 min. incubation using 4.3 µg enzyme protein as described in Chapter III.3.(i). A control reaction mixture was run without potassium phosphate in both the cases.

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TABLE VI

EFFECT OF SODIUM SULPHATE ON CEREBROSIDE 3-SULPHATE AND p-NITROCATECHOL SULPHATE DEGRADATION

Sulphate (mM)	Sulphate released from cerebroside 3-sulphate (nmol)	Nitrocatechol formed from nitrocatechol sulphate (nmol)
None	2.72	48
0.05	3.10	60
0.10	2.80	54
0.50	2.20	48
2.00	1.29	-
5.00	0.25	48

The assay mixture consisted of 0.01M-sodium acetate buffer pH 4.5, 70,000 c.p.m. (^{35}S)cerebroside 3-sulphate (22 nmol) in 0.2% Tween 20, indicated concentrations of sodium sulphate and 30 µg enzyme protein (0.76 unit) in a total volume of 0.1 ml. The reaction was terminated as indicated in the Methods.

The enzyme activity towards p-nitrocatechol sulphate was estimated for 5 min. incubation using 4.8 µg enzyme protein as described in Chapter III.3.(i). A control reaction mixture was run without sodium sulphate in both the cases.

species. Because the enzyme was heterogenous, a definite conclusion regarding the role of a heat-stable polymer for the degradation of cerebroside 3-sulphate cannot be made. However, even after the electrophoresis, the protein band corresponding to arylsulphatase A was able to degrade cerebroside 3-sulphate.

Recently Jatzkewitz (130) has reported that buffer concentration had a marked effect on enzyme activity when cerebroside 3-sulphate was used as substrate. There was increase in enzyme activity upto a final concentration of 0.01M but as the buffer concentration was increased to 0.02M, there was almost complete inhibition of enzyme activity. Further more "complementary heat-stable factor" is required only a high buffer concentration (0.2M) for cerebroside 3-sulphate degradation.

Like pig kidney cerebroside sulphatase, chicken arylsulphatase A has same pH value 4.5 and almost same Km value 0.12mM. It is interesting to note that with cerebroside 3-sulphate as substrate the enzyme does not show anomalous time-activity relationship as shown by the enzyme when p-nitrocatechol sulphate was used as substrate. Harinath and Robins (35) found with 4-methylumbelliferone sulphate as substrate, that the human brain arylsulphatase A did not show any anomalous

kinetics. These differences in the kinetic properties of arylsulphatase A using different substrates may be attributed to the nature of the substrate.

It is apparent from the result presented here that the chicken brain arylsulphatase A can desulphate cerebroside 3-sulphate. However, it may be noted that the relative rate at which this enzyme cleaves cerebroside 3-sulphate is 1600 times less than the rate at which it cleaves p-nitrocatechol sulphate. A similar observation has been made by Mehl and Jatzkewitz (79) in the case of pig kidney cerebroside sulphatase.

5. Summary:

Chicken brain arylsulphatase A can degrade cerebroside 3-sulphate without any "complementary heat-stable fraction." Like pig kidney cerebroside sulphatase the enzyme had pH optimum 4.5 in acetate buffer and the Km value for cerebroside 3-sulphate was 0.12mM. The enzyme showed linear time-activity relationship and inhibited by sulphite, phosphate and sulphate ions. The activity of chicken brain arylsulphatase A towards cerebroside 3-sulphate is stimulated by non-ionic detergents.

CHAPTER V.

GENERAL DISCUSSION AND SUMMARY

CHAPTER V.

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Although the widespread distribution of the arylsulphatases is well known, there is little information available on the amounts of these enzymes present in the various species, the only attempt at comparative studies being that of Roy (15) who reported the approximate relative proportions of arylsulphatases A and B in the liver of various animal species.

During our studies on the proportions of arylsulphatases A and B in brain of various animal species (Chapter II) it was found that there was a considerable variation in the concentration of these enzymes in the brain. It is interesting to note that there is relationship between mucopolysaccharide content and the amount of arylsulphatase B. Thus in rat, monkey and man, where mucopolysaccharide contents are very high (110), arylsulphatase B accounts for the greater part of the total arylsulphatase activity. On the other hand, in birds and sheep where mucopolysaccharide contents are low and cerebroside 3-sulphate concentration is high to that in rat and man (111), arylsulphatase A is present in higher amounts.

The results on the regional distribution of arylsulphatase A and B activities in monkey brain and in developing rat brain suggest a relationship between arylsulphatase A and cerebroside 3-sulphate and arylsulphatase B and mucopolysaccharides. These results are in agreement with the earlier observations from our laboratory by Balasubramanian and Pachhawat (42), Saxena et al (104), Kokrady et al (131) and Austin et al (103) who also found a striking relationship between arylsulphatase A and cerebroside 3-sulphate metabolism.

During our survey of arylsulphatase A and B activities in various animal species, it was observed that chicken brain arylsulphatase A is unique. It behaves like arylsulphatase B in its electrophoretic mobility, under zinc acetate fractionation, stimulation by Ag^{+} and inhibition by citrate ions. However, its kinetic properties such as Km value, anomalous kinetics, inhibitory effect of sulphite, phosphate and sulphate ions and molecular weight are similar to arylsulphatase A of other animal species.

Interest in arylsulphatases rose immensely after Austin et al (65,81) had proved the deficiency of arylsulphatase A in metachromatic leukodystrophy.

Mehl and Jatzkewitz (79) have recently reported that arylsulphatase A from pig kidney in the presence of a heat-stable factor is responsible for the degradation of cerebroside 3-sulphate. However, at present there is no information available as to whether or not brain arylsulphatase A can degrade cerebroside 3-sulphate. The chicken brain arylsulphatase A was purified to study its role and it was found that this enzyme can degrade cerebroside 3-sulphate in the absence of heat-stable factor. This degradation of cerebroside 3-sulphate without heat-stable polymer is noteworthy because this enzyme shows certain different properties compared to arylsulphatase A of other animal species.

The cerebroside 3-sulphate degrading activity of chicken brain arylsulphatase A is stimulated by non-ionic detergents. The enzyme has pH optimum at 4.5 in acetate buffer. The Km value for cerebroside 3-sulphate was 0.12mM which is comparable with 0.105mM Km value of pig kidney cerebroside 3-sulphatase. The enzyme is inhibited by sulphite, phosphate and sulphate ions and does not show anomalous kinetics as shown by arylsulphatase A of various animal species when p-nitrocatechol sulphate is used as substrate. Harinath and Robins (35) found that the human brain arylsulphatase A

did not show any anomalous kinetics with 4-methyl-umbelliferone sulphate as substrate. These differences in the kinetic properties of arylsulphatase A, using different substrates, may be attributed to the nature of the substrate.

To summarise, the present data clearly indicate that chicken brain arylsulphatase A can degrade cerebroside 3-sulphate.

Cerebroside 3-sulphate ⟶ cerebroside + sulphate

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APPENDIX

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ENZYMATIC DEPHOSPHORYLATION OF 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE TO ADENOSINE 5'-PHOSPHOSULFATE IN SHEEP BRAIN

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SUMMARY

An enzyme catalyzing the dephosphorylation of 3'-phosphoadenosine 5'-phospho[^{35}S]sulfate to adenosine 5'-phospho[^{35}S]sulfate was partially purified from sheep brain. The enzyme showed an optimum pH of 5.0; it was activated by EDTA and inhibited by the divalent metal ions tested. ADP and 3'-AMP had no significant influence on the enzyme activity, but 3'-phosphoadenosine 5'-phosphate was a potent inhibitor. NaF completely inhibited the reaction. The enzyme exhibited properties much different from those of 3'-nucleotidase and 3'-phosphoadenosine 5'-phosphosulfate sulfohydrolase of brain. The physiological role of the phosphohydrolase may be in the regulation of the concentration of 3'-phosphoadenosine 5'-phosphosulfate.

INTRODUCTION

3'-Phosphoadenosine 5'-phosphosulfate (PAPS) is recognized to be the biological sulfate donor in the formation of several sulfated compounds[1]. The enzymatic synthesis of PAPS and the transfer of sulfate from PAPS to mucopolysaccharides and sulfatides in brain was demonstrated in earlier work from this laboratory[2–4]. The enzymatic degradation of PAPS to inorganic sulfate was observed in several mammalian tissue extracts[5–8], and the properties of this enzyme, PAPS sulfohydrolase from sheep brain was described earlier[5].

The formation of adenosine 5'-phosphosulfate (APS) as a degradation product of PAPS was reported by SPENCER[6] in rat-liver supernatant and by SUZUKI AND STROMINGER[7] and HARADA *et al.*[9] in hen oviduct preparations. ROBBINS AND LIPMANN[10] used a 3'-nucleotidase (3'-ribonucleotide phosphohydrolase, EC 3.1.3.6) from rye grass for the enzymatic conversion of PAPS to APS. It was observed[11] that free APS as an intermediate in the synthesis of PAPS is not formed in significant quantities especially because of the highly unfavorable equilibrium of the ATP sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) reaction in the direction of formation

Abbreviations: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

of APS and also because of the high affinity of the enzyme APS kinase (ATP:adenylylsulfate 3'-phosphotransferase, EC 2.7.1.25) for APS. Furthermore it has been shown in this laboratory[12] that APS remains bound to a protein during its enzymatic synthesis by a purified preparation of ATP sulfurylase. It would appear that the major route by which free APS is formed is by the enzymatic dephosphorylation of PAPS. The present report is concerned with studies on the enzymatic conversion of PAPS to APS catalyzed by a partially purified PAPS phosphohydrolase from sheep brain. Evidences are also presented to indicate that the enzyme differs from brain PAPS sulfohydrolase and 3'-nucleotidase in several of its characteristics.

MATERIALS AND METHODS

Carrier free $^{35}SO_4^{2-}$ was obtained from Atomic Energy Establishment (Trombay, India). 3'-Phosphoadenosine 5'-phosphate was a gift from Dr. J. H. Austin. All other nucleotides were from Sigma Chemical Co. (U.S.A.). DEAE-cellulose (Selectacel reagent) and DEAE-Sephadex A-25 were products of Carl Schleicher and Schuell Co. (U.S.A.) and Pharmacia Fine Chemicals (Sweden) respectively. DEAE-cellulose was washed sequentially with 1 M NaOH, water, 1 M HCl, water, 1 M NaOH and finally with water before equilibration with the buffer. DEAE-Sephadex was allowed to swell in excess of water, washed sequentially with 0.5 M HCl, water, 0.5 M NaOH and finally with water before equilibration with the buffer.

Preparation of APS. APS was prepared according to the method of BADDILEY *et al.*[13] from pyridine sulfur trioxide and AMP. APS was isolated by descending chromatography on Whatman No. 3 paper using the solvent system ethanol–1 M ammonium acetate (7.5:3, v/v). It was eluted from the paper with 0.02 M Tris–HCl buffer (pH 8.5) at 4° and concentrated under vacuum at 30°.

Preparation of [^{35}S]PAPS. Carrier-free [^{35}S]PAPS was synthesized using an enzyme preparation from rat liver. The enzyme was prepared as described by BRUNNGRABER[14] with some modifications. 8 g of the liver were homogenized with 24 ml of 1.15% KCl solution and centrifuged at 20 000 × *g* for 1 h at 4°. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, and the precipitate obtained between 1.5 and 2.1 M concentration of $(NH_4)_2SO_4$ was dissolved in the minimum amount of 0.02 M Tris–HCl buffer (pH 7.4) and was desalted by passing through a Sephadex G-75 column (2.2 cm × 22 cm) previously equilibrated with the same buffer. The elution was carried out with the same buffer and the fractions at the void volume were collected, pooled and used for [^{35}S]PAPS synthesis. The incubation mixture for the synthesis of [^{35}S]-PAPS consisted of 20 μmoles of Tris–HCl buffer (pH 8.0), 1 μmole of $MgCl_2$, 30 μC of carrier free $^{35}SO_4^{2-}$, 1.5 μmoles of ATP, 1 μmole of cysteine and 0.04 ml of the enzyme fraction in a total volume of 0.15 ml. [^{35}S]PAPS was isolated from the reaction mixture by chromatography as described earlier[3]. The [^{35}S]PAPS thus prepared contained about 0.3% of [^{35}S]APS and 15% $^{35}SO_4^{2-}$.

Assay of the PAPS phosphohydrolase. The assay mixture contained 30 μmoles of sodium acetate buffer (pH 5.0), [^{35}S]PAPS (about 600 000 counts/min corresponding to 1.9 pmoles) and the enzyme in a total volume of 0.15 ml. After incubation for 1 h at 37°, the reaction was stopped by heating in a boiling-water bath for 1 min. Immediately after heating, the mixture was cooled in ice and the precipitated protein was removed by centrifugation. An aliquot of the supernatant was streaked in the form of

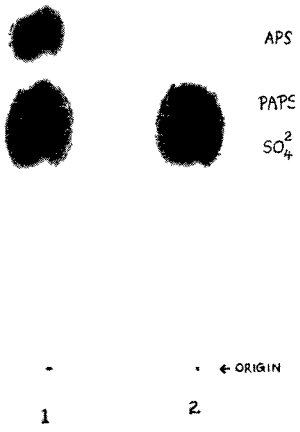


Fig 1 Radioautogram of the chromatographic separation of [^{35}S]APS from [^{35}S]PAPS and $^{35}SO_4^{2-}$ Conditions of assay were the same as described in the text 1 Reaction mixture containing 30 μmoles of sodium acetate buffer (pH 5 0) 600 000 counts/min of [^{35}S]PAPS and 0 03 mg of purified enzyme protein 2 Control reaction mixture

a 1-cm band on Whatman No 3 paper Descending chromatography was carried out using the solvent system[7] isobutyric acid–0 5 N ammonia (5 3, v/v) (Fig 1) The area corresponding to [^{35}S APS was cut out from the paper and the radioactivity was measured in a Nuclear Chicago scintillation counter A control reaction mixture in which the enzyme was added at the end of the incubation period was run simultaneously Enough [^{35}S]PAPS was used in all reaction mixtures, and unreacted [^{35}S]PAPS could be detected at the end of the reaction in all the enzyme assays The radioactivity of [^{35}S]APS was corrected for absorption by the paper

Assay for 3'-nucleotidase The reaction mixture consisted of 50 μmoles of sodium acetate buffer (pH 5 0), 0 5 μmole of 3'-AMP, 0 8 μmole of $CoCl_2$ and the enzyme in a total volume of 0 3 ml After incubation for 30 min at 37°, 1 ml of 10% trichloroacetic acid was added to the mixture, the precipitated protein was removed by centrifugation and P_i released was estimated by the method of FISKE AND SUBBAROW[15]

PAPS sulfohydrolase was assayed as described by BALASUBRAMANIAN AND BACHHAWAT[5]

Protein was estimated according to LOWRY *et al* [16]

Preparation of PAPS phosphohydrolase All operations were carried out at 0–4°

Extraction Fresh sheep brain cut into small pieces (100 g) was homogenized with 200 ml of 0 1 M Tris HCl buffer (pH 7 4) in an ultra-Turrax homogenizer The

homogenate was centrifuged at 12 000 × *g* for 20 min, and the supernatant was dialyzed against 1 l of 0.1 M Tris–HCl (pH 7.4) for 4 h.

$(NH_4)_2SO_4$ fractionation. The dialyzed supernatant was brought to an $(NH_4)_2SO_4$ saturation of 40% by the addition of solid $(NH_4)_2SO_4$ with stirring and centrifuged at 12 000 × *g* for 20 min. The precipitate was discarded. The supernatant was further treated with $(NH_4)_2SO_4$ to a saturation of 60% and centrifuged at 12 000 × *g* for 20 min. The precipitate was dissolved in 12 ml of 0.01 M Tris–HCl buffer (pH 7.4) and dialyzed against 500 ml of the same buffer for 4 h.

DEAE-cellulose chromatography. The dialyzed preparation was applied on a DEAE-cellulose column (15 cm × 1.5 cm) equilibrated with 0.01 M Tris–HCl (pH 7.4). The column was washed with 200 ml of the same buffer, and fractions of 15 ml were collected. The first five fractions containing enzyme activity were pooled and $(NH_4)_2SO_4$ was added to 90% saturation. The precipitate was collected by centrifugation and dissolved in 1 ml of 0.01 M Tris–HCl (pH 7.4) and dialyzed against 500 ml of the same buffer overnight.

DEAE-Sephadex chromatography. The DEAE-cellulose fraction was applied on a DEAE-Sephadex column (6.5 cm × 0.7 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 7.4). The column was washed with 20 ml of the same buffer and to the washings $(NH_4)_2SO_4$ was added to 90% saturation. The precipitate was collected by centrifugation, dissolved in 1 ml of 0.01 M Tris–HCl (pH 7.4) and dialyzed against 500 ml of the same buffer overnight.

RESULTS

Purification of the enzyme

Table I shows a summary of the purification of the enzyme. The procedure resulted in a 13-fold increase in specific activity of the enzyme with a recovery of 4% of the original activity. Modifications of the procedure or other processes of purification, involving calcium phosphate gel treatment, heat treatment or acid precipitation, were tried without success as they did not improve upon the recovery or the increase in specific activity. Elution with various concentrations of NaCl resulted in the recovery of smaller quantities of the enzyme from the DEAE-cellulose and DEAE-

TABLE I

PURIFICATION OF PAPS PHOSPHOHYDROLASE

Conditions of assay were the same as described in the text.

Fraction	*Volume (ml)*	*Total units* × 10³*	*Total protein (mg)*	*Specific activity (units/mg protein) × 10⁶*	*Yield (%)*
Crude (12 000 × *g* supernatant)	165	1.15	1423	0.81	100
$(NH_4)_2SO_4$ fraction	13	0.275	172	1.6	24
DEAE-cellulose fraction	2.5	0.067	10	6.7	6
DEAE-Sephadex fraction	2.5	0.049	4.5	10.9	4

* One enzyme unit is defined as 1 μmole of [^{35}S]APS formed in 1 h.

Sephadex columns; however, the specific activity was decreased in the enzyme fractions. The major loss in the recovery of the enzyme occurred in the $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography steps. The discarded fractions in these steps exhibited some PAPS phosphohydrolase activity of much lower specific activity. The cause for the enzyme loss is not clearly understood, but it could result from the enrichment of certain interfering factors such as PAPS sulfohydrolase in these steps during the course of purification.

The purified enzyme exhibited about 78% of its original activity after storage at −18° for 2 weeks.

Identification of the product of the reaction

The product of the reaction was identified as [^{35}S]APS by paper chromatography and electrophoresis. The radioactive reaction product in the chromatographic assay was eluted from the paper by water at 4° and concentrated under vacuum. The reaction product and the authentic sample of APS had the same R_F value (0.36) in the solvent system isobutyric acid–0.5 N ammonia (5:3, v/v). Cochromatography of the radioactive reaction product and authentic sample of APS on Whatman No. 3 paper in the solvent system ethanol–1 M ammonium acetate (7.5:3, v/v) showed that the radioactivity coincided with the ultraviolet-quenching area of authentic APS (Fig. 2).

Electrophoresis on a 7.5 cm × 37.5 cm strip of Whatman No. 3 paper was carried out using a mixture of equal volumes[7] of 0.1 M Tris–acetate buffer (pH 6.5) and 0.05 M

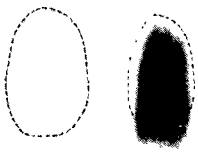


Fig. 2. Paper chromatography of the radioactive product and authentic sample of APS. Dotted lines outline the ultraviolet-quenching area of authentic APS, and the dark spot indicates the radioactive product. 1. Authentic APS. 2. A mixture of authentic APS and radioactive product.

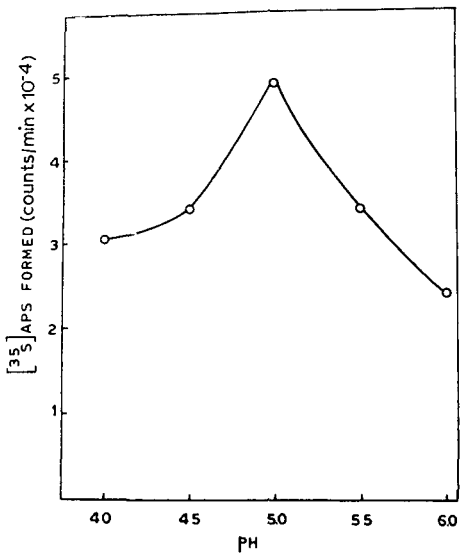
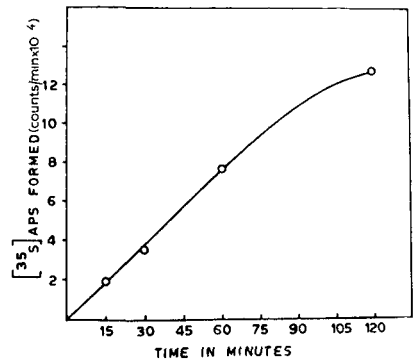


Fig 3 pH optimum of PAPS phosphohydrolase Assay conditions were the same as described in the text except that sodium acetate buffer of various pH's were used as shown in the figure. [^{35}S]PAPS 600 000 counts/min and 0 03 mg enzyme were used

citrate–phosphate buffer (pH 6 5) for 2 h at 600 V Under these conditions the distances moved by the radioactive product, [^{35}S]PAPS and $^{35}SO_4^{2-}$ were 10, 14 and 23 5 cm, respectively The mobility of the radioactive product was the same as that of authentic sample of APS

pH optimum and time-course of the reaction

The optimal pH for the enzymatic dephosphorylation of PAPS was pH 5 0 (Fig. 3) The [^{35}S]APS formation showed a linear increase with time up to a period of 60 min, under the usual conditions of assay (Fig 4)



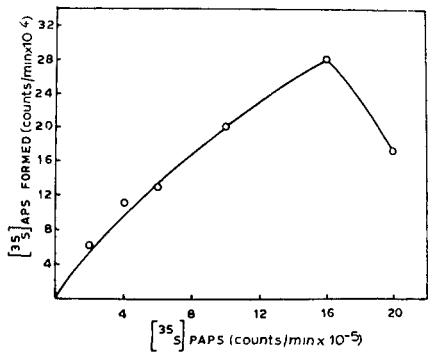


Fig 4 The time course of [^{35}S]APS formation Assay conditions were the same as described in the text except that incubation period was varied as shown in the figure [^{35}S]PAPS 600 000 counts/min and 0 007 mg of enzyme protein were used

Fig 5 Effect of substrate concentration on [^{35}S]APS formation Assay conditions were the same as described in the text except that [^{35}S PAPS concentration was varied as indicated in the figure 0 007 mg of enzyme was used

TABLE II

EFFECT OF METAL IONS AND EDTA ON PAPS PHOSPHOHYDROLASE

Assay mixture consisted of 2 μmoles of each addition, 30 μmoles of acetate buffer (pH 5 5), 600 000 counts/min of [^{35}S]PAPS and 0 09 mg of enzyme protein in a total volume of 0 15 ml. Assay conditions were the same as described in the text

Addition	*[^{35}S]APS formed (counts/min)*
None	58 660
EDTA	98 460
$MgCl_2$	46 710
$CoCl_2$	12 510
$MnCl_2$	7 330

Effect of substrate concentration

The variation of [^{35}S]APS formation with increasing amounts of [^{35}S]PAPS is shown in Fig. 5. There was an increase up to 1.6×10^6 counts/min of [^{35}S]- PAPS used. However, with $2.0 \cdot 10^6$ counts/min of [^{35}S]PAPS, there was a sharp decrease in enzyme activity, and with further increase in [^{35}S]PAPS, the activity declined.

EDTA and metal ion requirements of PAPS phosphohydrolase

Metal ions such as Mg^{2+}, Co^{2+} and Mn^{2+} were inhibitory, but EDTA stimulated the reaction (Table II). In contrast, the PAPS sulfohydrolase of brain has been reported to be activated by Co^{2+} and Mn^{2+} (ref. 5). 3'-Nucleotidase activity of brain was found to be stimulated by Co^{2+}, Mg^{2+} and Mn^{2+}, as described below.

Effect of nucleotides and other substances on PAPS phosphohydrolase

The effect of some nucleotides, –SH compounds and NaF on the enzyme activity is shown in Table III. ADP and 3'-AMP were without significant influence on the enzyme activity. ADP is known to be a powerful inhibitor of brain PAPS sulfohydrolase[5].

TABLE III

EFFECT OF NUCLEOTIDES AND OTHER SUBSTANCES ON PAPS PHOSPHOHYDROLASE

Assay mixture consisted of 30 μmoles of acetate buffer (pH 5 5), 2 μmoles of each addition, 600 000 counts/min of [^{35}S]PAPS and 0.09 mg of enzyme protein. Assay conditions are described in the text.

Addition	*[^{35}S]APS formed (counts/min)*
None	53 320
ADP	51 300
3'-AMP	51 310
3'-Phosphoadenosine 5'-phosphate	24 300
GSH	48 140
2,3-Dimercaptopropanol	43 100
NaF	0

3'-Phosphoadenosine 5'-phosphate was a powerful inhibitor of the enzyme The inhibition may be due to the close structural similarity of 3'-phosphoadenosine 5'-phosphate to PAPS GSH and 2,3-dimercaptopropanol depressed the enzyme activity NaF completely inhibited the reaction

3'-Nucleotidase and PAPS sulfohydrolase of brain

3'-Nucleotidase and PAPS sulfohydrolase activities were present in the purified enzyme preparation The recovery of these two enzyme activities from the crude extract was 0 4 and 0 02%, respectively, as compared to the value of 4% for the PAPS phosphohydrolase Since it has been reported[10] that 3'-nucleotidase can convert PAPS to APS, the properties of this enzyme activity in the purified fraction were studied in detail, using 3'-AMP as substrate The nucleotidase showed an optimum pH of 5 0 Metal ions like Co^{2+}, Ni^{2+}, Mn^{2+} and Mg^{2+} markedly stimulated the enzyme activity, and EDTA was slightly inhibitory (Table IV) This observation points to the possibility that the 3'-nucleotidase may be different from the PAPS phosphohydrolase In support of this idea was the observation that 3'-AMP did not inhibit the PAPS phosphohydrolase activity (Table III)

The PAPS phosphohydrolase of sheep brain exhibits widely different properties when compared to the PAPS sulfohydrolase also The latter enzyme has an optimum pH of 6 0, it is markedly activated by Co^{2+} and strongly inhibited by ADP (ref 5)

During the purification of the enzyme from the crude extract, the specific activity of PAPS phosphohydrolase increased to about 13 fold in the purified DEAE-Sephadex fraction However, the specific activity of PAPS sulfohydrolase decreased and that of 3' nucleotidase increased to only about 1 5 times in the purified preparation

TABLE IV

EFFECT OF METAL IONS AND EDTA ON 3' NUCLEOTIDASE

The reaction mixture consisted of 50 μmoles of sodium acetate buffer (pH 5) 0 5 μmole of 3'-AMP 0 5 μmole of each addition and 0 22 mg of enzyme protein in a total volume of 0 3 ml Assay was done as described in the text

Addition	*Phosphate liberated (μmoles)*
None	0 010
$CoCl_2$	0 065
$NiCl_2$	0 035
$MgCl_2$	0 035
$MnCl_2$	0 029
EDTA	0 016

The ratio of specific activities of 3'-nucleotidase and PAPS sulfohydrolase to the specific activity of the phosphohydrolase in the crude and purified enzyme fractions is shown in Table V It is seen that the values are much lower for the purified enzyme fraction, compared to the values for the crude extract These considerations evidently point out that the PAPS phosphohydrolase exhibits characteristics much different from those of the 3'-nucleotidase and PAPS sulfohydrolase and indicate the possibility that different enzymes are responsible for these three activities in the brain

TABLE V

THE RATIO OF SPECIFIC ACTIVITIES OF PAPS PHOSPHOHYDROLASE, PAPS SULFOHYDROLASE AND 3′-NUCLEOTIDASE IN THE CRUDE AND PURIFIED ENZYME FRACTIONS

The purification procedure of the enzyme was as described in the text

Enzyme fraction	*Ratio of specific activities*	
	*3′-Nucleotidase** / *PAPS phosphohydrolase***	*PAPS sulfohydrolase**** / *PAPS phosphohydrolase*
Crude (12 000 × *g* supernatant)	0.617	1.09
DEAE-Sephadex fraction	0.073	0.01

* Assay was done as described in the text. Unit of activity was expressed as one μmole of phosphate liberated from 3′-AMP in 30 min.

** Assay was as described in the text. Unit of activity and specific activity were as given in Table I. There was about 13 fold increase in specific activity in the DEAE Sephadex fraction compared to the crude extract.

*** Assay was as described in the text. Unit of activity was defined as 1 μmole of $^{35}SO_4^{2-}$ liberated from [^{35}S]PAPS in 1 h.

DISCUSSION

The present study demonstrates the conversion of PAPS to APS catalyzed by a partially purified enzyme preparation from sheep brain. Although the purified PAPS phosphohydrolase preparation has some 3′-nucleotidase and PAPS sulfohydrolase activity, a study of the pH optimum, the influence of metal ions and the effect of nucleotides and other substances show that these three enzyme activities differ from each other in their characteristics and indicate the possibility that different enzymes are responsible for these three activities. Furthermore, the specific activity of the PAPS phosphohydrolase had increased about 13-fold from the crude to the purified enzyme preparation, but there was practically no increase in the specific activities of the PAPS sulfohydrolase and 3′-nucleotidase.

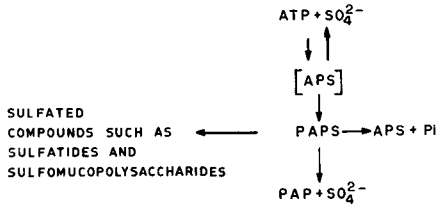


Fig. 6. Reactions leading to the synthesis and degradation of PAPS and sulfate transfer from PAPS in brain. PAP — 3′-phosphoadenosine 5′-phosphate.

From the present studies and the earlier work done in this laboratory[2–5], it is apparent that the concentration of PAPS is regulated by several enzymes in the brain. The various reactions catalyzed by these enzymes is shown in Fig. 6. While the importance of PAPS synthesis and sulfate transfer from PAPS to various acceptors is understandable, the significance of the PAPS-degrading enzymes such as PAPS

sulfohydrolase and PAPS phosphohydrolase is not quite clear. The physiological role of these degrading enzymes may lie in the regulation of the concentration of PAPS or some of its degraded products such as APS, inorganic sulfate or 3'-phosphoadenosine 5'-phosphate. The presence of a rat-liver sulfohydrolase enzyme acting on adenylylsulfate was recently reported by BAILEY-WOOD *et al.*[17]. We have also observed in preliminary experiments a weak sulfohydrolase activity in the purified brain enzyme preparation with [^{35}S]APS as substrate (pH 6.0).

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